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(54) Title: METHODS OF DOWNMODULATING THE IMMUNE RESPONSE TO THERAPEUTIC PROTEINS

(57) Abstract

Compositions and methods for treating a hemostatic disorder using agents which promote hemostasis and agents which inhibit a costimulatory signal in a T cell are provided. The instant compositions and methods enable the treatment of hemostatic disorders using foreign therapeutic proteins, while downmodulating immune responses to the therapeutic proteins.

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METHODS OF DOWNMODULATING THE IMMUNE RESPONSE TO THERAPEUTIC PROTEINS

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Background of the Invention

One of the primary limitations of therapeutic treatment using biological proteins is the immune response which the body generates in response to the presence of foreign substances in the body. This immune response is especially problematic when foreign substances must be repeatedly administered in order to be optimally effective.

One example of such a situation is the repeated administration of agents for the treatment of hemostatic disorders, such as the factor VIII deficiency diseases (e.g., classic hemophilia A and von Willebrand's disease) or factor IX deficiency, also known as hemophilia B. Classic hemophilia, hemophilia A, is an X-linked disorder which affects 1 in 10,000 males. Von Willebrand's disease is the most common inherited bleeding disorder, occurring in as many as 1 in 800 to 1000 individuals. Hemophilia B, also known as Christmas disease, occurs in roughly 1 in 100,000 males (*Harrison's Principles of Internal Medicine*. Isselbacher et al., eds. 13th Edition. 1994. McGraw-Hill N.Y., N.Y.).

Factor VIII is a 265 kD single chain protein which circulates in a complex with von Willebrand factor (VWF). Factor VIII is an important regulatory protein in the blood coagulation cascade. After activation by thrombin, it accelerates the rate of factor X activation by activated factor IX (factor IXa), eventually leading to the formation of the fibrin clot. The VWF molecule is an adhesive glycoprotein that plays a central role in platelet agglutination. It serves as a carrier for factor VIII in plasma and facilitates platelet-vessel wall interactions. VWF is made up of multiple, probably identical, subunits each of about 230 kD. VWF is synthesized in endothelial cells and megakaryocytes. Factor IX is a single-chain 55 kD proenzyme which is converted to an

active protease (IXa) by factor XIa or by tissue factor-VIIa complex. Activated factor IX and activated factor VIII then activate factor X.

Repeated administration of foreign proteins can lead to an immune response to those proteins in a recipient. In the T cell response to foreign proteins two signals must be provided by antigen-presenting cells (APCs) to resting T lymphocytes (Jenkins, M. and Schwartz, R. (1987) *J. Exp. Med.* 165, 302-319; Mueller, D.L., et al. (1990) *J. Immunol.* 144, 3701-3709). The first signal, which confers specificity to the immune response, is transduced via the T cell receptor (TCR) following recognition of foreign antigenic peptide presented in the context of the major histocompatibility complex (MHC). The second signal, termed costimulation, induces T cells to proliferate and become functional (Lenschow et al. 1996. *Annu. Rev. Immunol.* 14:233). Costimulation is neither antigen-specific, nor MHC restricted and is thought to be provided by one or more distinct cell surface molecules expressed by APCs (Jenkins, M.K., et al. 1988 *J. Immunol.* 140, 3324-3330; Linsley, P.S., et al. 1991 *J. Exp. Med.* 173, 721-730; Gimmi, C.D., et al., 1991 *Proc. Natl. Acad. Sci. USA.* 88, 6575-6579; Young, J.W., et al. 1992 *J. Clin. Invest.* 90, 229-237; Koulova, L., et al. 1991 *J. Exp. Med.* 173, 759-762; Reiser, H., et al. 1992 *Proc. Natl. Acad. Sci. USA.* 89, 271-275; van-Seventer, G.A., et al. (1990) *J. Immunol.* 144, 4579-4586; LaSalle, J.M., et al., 1991 *J. Immunol.* 147, 774-80; Dustin, M.I., et al., 1989 *J. Exp. Med.* 169, 503; Armitage, R.J., et al. 1992 *Nature* 357, 80-82; Liu, Y., et al. 1992 *J. Exp. Med.* 175, 437-445).

The CD80 (B7-1) and CD86 (B7-2) proteins, expressed on APCs, are critical costimulatory molecules (Freeman et al. 1991. *J. Exp. Med.* 174:625; Freeman et al. 1989 *J. Immunol.* 143:2714; Azuma et al. 1993 *Nature* 366:76; Freeman et al. 1993. *Science* 262:909).

B7-2 appears to be more significant during primary immune responses, while B7-1, which is upregulated later in the course of an immune response, may be important in prolonging primary T cell responses or costimulating secondary T cell responses (Bluestone. 1995. *Immunity.* 2:555).

B7-1 and B7-2 are the counter-receptors for two ligands expressed on T lymphocytes. One ligand to which B7-1 and B7-2 bind, CD28, is constitutively expressed on resting T cells and increases in expression after activation. After signaling through the T cell receptor, ligation of CD28 and transduction of a costimulatory signal

induces T cells to proliferate and secrete IL-2 (Linsley, P.S., et al. 1991 *J. Exp. Med.* 173, 721-730; Gimmi, C.D., et al. 1991 *Proc. Natl. Acad. Sci. USA.* 88, 6575-6579; June, C.H., et al. 1990 *Immunol. Today.* 11, 211-6; Harding, F.A., et al. 1992 *Nature.* 356, 607-609.). The second ligand, termed CTLA4 (CD152) is homologous to CD28 but is not expressed on resting T cells and appears following T cell activation (Brunet, J.F., et al., 1987 *Nature* 328, 267-270). CTLA4 appears to be critical in negative regulation of T cell responses (Waterhouse et al. 1995. *Science* 270:985). Blockade of CTLA4 has been found to remove inhibitory signals, while aggregation of CTLA4 has been found to provide inhibitory signals that downregulate T cell responses (Allison and Krummel. 1995. *Science* 270:932). The B7 molecules have a higher affinity for CTLA4 than for CD28 (Linsley, P.S., et al., 1991 *J. Exp. Med.* 174, 561-569) and B7-1 and B7-2 have been found to bind to distinct regions of the CTLA4 molecule and have different kinetics of binding to CTLA4 (Linsley et al. 1994. *Immunity.* 1:793).

Between 10 and 25 percent of patients with hemophilia develop an immune response to Factor VIII. These patients develop inhibitors, usually IgG antibodies, which neutralize factor VIII activity and, thus, prevent effective therapy. Two types of inhibitors have been identified. High responder patients with type I inhibitors have an anamnestic response to factor VIII which results in an increased titer of antibodies to factor VIII. Low responder patients with type II inhibitor have a low antibody titer which is not increased by administration of factor VIII. Current strategies to blunt the antibody response in these patients have only been marginally successful. Moreover, the development of antibodies to replaced proteins is a critical problem that needs to be solved if gene therapy is to be successful in the treatment of hemophilias and other deficiency diseases (Connelly S et al, Blood 88:3846, 1996; Kuna S-H et al, Blood 91:784, 1998).

Summary of the Invention

The present invention provides, inter alia, compositions and methods which allow the administration of a therapeutic protein to treat a disorder while reducing the development and/or progression of an immune response to the therapeutic protein.

In one aspect, the invention pertains to compositions comprising a first agent which promotes hemostasis and a second agent which inhibits a costimulatory signal in a T cell.

In one embodiment, the subject compositions further comprise a pharmaceutically acceptable carrier.

In one embodiment, the first agent is factor VIII. In another embodiment, the first agent is a B-domain deleted variant of factor VIII. In one embodiment, the first agent is factor IX. In another embodiment, the first agent is Von Willebrand factor.

In one embodiment, the second agent is a soluble form of a costimulatory molecule. In a preferred embodiment, the second agent is a soluble form of CTLA4. In another preferred embodiment, the second agent is a soluble form of B7-1, a soluble form of B7-2, or a combination of a soluble form of B7-1 and a soluble form of B7-2. In a more preferred embodiment, the second agent is CTLA4Ig. In another more preferred embodiment, the second agent is B7-1Ig or B7-2Ig. In yet another preferred embodiment, the second agent is a soluble form of CD40 or CD40L.

In another embodiment, the second agent is an antibody which binds to a costimulatory molecule. In a preferred embodiment, the second agent is selected from the group consisting of an anti-B7-1 antibody, anti-B7-2 antibody, and a combination of an anti-B7-1 and an anti-B7-2 antibody. In one embodiment, the antibody is a non-activating form of an anti-CD28 antibody.

The invention further pertains to methods of treating a hemostatic disorder in a subject comprising administering to a subject the instant compositions such that a hemostatic disorder is treated.

In one embodiment, the subject has a significant titer of antibodies which bind to the first agent. In another embodiment, the subject does not have a significant titer of antibodies which bind to the first agent.

In one embodiment, the methods comprise administering a composition comprising an agent which inhibits a costimulatory signal in a T cell.

In one embodiment, the hemostatic disorder is selected from the group consisting of hemophilia A, hemophilia B, and von Willebrand's disease.

In another aspect, the invention pertains to methods of treating a hemostatic disorder in a subject comprising administering to the subject a first agent which promotes hemostasis and a second agent which inhibits a costimulatory signal in a T cell, such that a hemostatic disorder is treated.

In another aspect, the invention pertains to methods of treating a hemostatic disorder in a subject comprising administering to the subject a first agent which promotes hemostasis and a second agent which inhibits a costimulatory signal in a T cell, such that immunotolerance to the first agent occurs thereby treating a hemostatic disorder.

In one embodiment, the first agent is factor VIII. In another embodiment, the first agent is a B-domain deleted variant of factor VIII. In another embodiment, the first agent is factor IX. In another embodiment, the first agent is Von Willebrand factor.

In one embodiment, the second agent is a soluble form of an agent which delivers a costimulatory signal to a T cell. In a preferred embodiment, the agent is a soluble form of CTLA4. In a more preferred embodiment, the agent is CTLA4Ig. In another preferred embodiment, the agent is a soluble form of B7-1, a soluble form of B7-2 or a combination of both B7-1 and B7-2. In another more preferred embodiment, the agent is B7-1Ig, B7-2Ig, or a combination of both B7-1Ig and B7-2Ig.

In one embodiment, the second agent is an antibody which binds to a costimulatory molecule. In another embodiment, the second agent is selected from the group consisting of an anti-B7-1 antibody, an anti-B7-2 antibody, and a combination of an anti-B7-1 and an anti-B7-2 antibody. In another embodiment, the antibody is a non-activating form of an anti-CD28 antibody.

In one embodiment, the hemostatic disorder is selected from the group consisting of hemophilia A, hemophilia B, and von Willebrand's disease.

In one embodiment, the subject has a significant titer of antibodies which bind to the first agent.

Brief Description of the Drawings

Figure 1 illustrates the experimental design used for Example 1 to test the inhibition of primary antibody responses to factor VIII.

Figure 2 shows that while mice that did not receive CTLA4Ig had high titers of antibody beginning as early as day 20 (G-1), mice that received CTLA4Ig did not develop antibodies until day 82 (G-2 and G-3).

Figure 3 illustrates the experimental design used for Example 2 to test the inhibition of secondary antibody responses to factor VIII.

Figure 4 shows that animals that did not receive CTLA4Ig had high titers of anti-factor VIII antibodies (G-1), while the mice that received CTLA4Ig, (G-2),with the exception of 1 mouse, did not develop a secondary immune response to factor VIII.

Figure 5 shows the effect of mCTLA4-Ig on anti-factor VIII antibody formation.

Figure 6 shows the effect of repeated administration of mCTLA4-Ig on anti-factor VIII antibody formation.

Figure 7 shows the effect of simultaneous administration of mCTLA4-Ig and factor VIII.

Figure 8 shows the effect of mCTLA4-Ig on the secondary immune response to factor VIII.

Figure 9 shows the role of B7- 1 and B7-2 in the anti-factor VIII antibody response.

Figure 10 shows the T cell response to factor VIII for hemophilia A/B7-$1^{-/-}$ and hemophilia A/B7-$2^{-/-}$ mice.

Detailed Description

The present invention represents an important advance in the treatment of hemostatic disorders by providing compositions and methods which allow

administration of a therapeutic protein to treat a disorder while reducing the development and/or progression of an immune response to the therapeutic protein.

Before further description of the invention, certain terms employed in the specification, examples and appended claims are, for convenience, collected here.

I. Definitions

As used herein, the language "hemostatic disorder" includes disorders which result in abnormal bleeding and/or thrombosis. Normal hemostasis limits blood loss by a series of interactions between components of blood vessel walls, platelets, and plasma proteins. Hemostatic disorders occur, for example, due to a failure in platelet aggregation and/or fibrin clot formation which can result in inappropriate responses to disease or trauma, e.g., uncontrolled bleeding. Such disorders can be detected, e.g., by determining bleeding time, partial thromboplastin time (PTT), prothrombin time (PT), thrombin time (TT), or by a quantitative fibrinogen determination using methods well known in the art. Exemplary hemostatic disorders include hemophilia A, hemophilia B, and von Willebrand's disease.

As used herein, the language "agent which promotes hemostasis" includes a protein or polypeptide which is deficient or deleted in a subject and which, when administered to the subject, ameliorates or treats a hemostatic disorder. Preferred agents which promote hemotstasis include coagulation factors such as Factor VIII, Factor IX, VWF and analogs thereof.

The term "B7 family" or "B7 molecules" as used herein includes costimulatory molecules that share amino acid sequence identity with B7 polypeptides, e.g., with B7-1, B7-2, or B7-3 (recognized by the antibody BB-1). In addition, the B7 family of molecules share a common function, e.g., the ability to bind to a B7 family ligand (e.g., one or more of CD28, CTLA4, or ICOS) and the ability to costimulate T cell activation.

B7 polypeptides are capable of providing costimulation to activated T cells to thereby induce T cell proliferation and/or cytokine secretion or of inhibiting costimulation of T cells, e.g., when present in soluble form. B7 family members include B7-1, B7-2 , and soluble fragments or derivatives thereof. In one embodiment, B7 family members bind to CTLA4, CD28, ICOS, and/or other ligands on immune cells and have the ability to inhibit or induce costimulation of immune cells.

As used herein, the language "agent which inhibits a costimulatory signal in a T cell" includes agents which inhibit a signal generated by the interaction of a costimulatory molecule on an antigen presenting cell (APC), e.g., a B7 family molecule and its counter receptor on a T cell. Costimulatory molecules on APCs (e.g., B7 family members) and their cognate ligands on T cells (e.g., CTLA4, CD28, and ICOS) are herein collectively referred to as costimulatory molecules. An agent which inhibits a costimulatory signal can act either extracellularly to inhibit the interaction between costimulatory molecules, thus blocking the production of intracellular signals, or can act intracellularly to inhibit costimulatory signals in a signal transduction pathway. Exemplary agents are described in further detail herein and include, for example, soluble forms of costimultory molecules and antibodies which bind to costimulatory molecules.

As used herein, the phrase "downmodulation of the immune response" includes reduction in an immune response (e.g., suppression, dampening, or inhibition) in a patient that does not have an existing immune response or reduction in the length and magnitude of an existing immune response. The term "immune response" includes any type of immune response which is initiated by or dependent upon costimulatory signals, e.g., a cellular or a humoral response, that can occur in a subject in response to a foreign antigen. In one embodiment the immune response is an antibody response to an agent which promotes hemostasis (e.g., Factor VII, VWF, or Factor IX). The term "immunotolerization" includes the induction of antigen specific tolerance which can be measured using techniques that are known in the art, e.g., by measuring secondary immune responses (e.g., cellular or humoral responses) to an antigen.

II. Agents Which Promote Hemostasis

In one embodiment, the agent which promotes hemostasis is factor VIII. The term "Factor VIII", as used herein, includes proteins exhibiting procoagulant activity characteristic of factor VIII. In one embodiment of the invention, factor VIII proteins are naturally occurring factor VIII proteins. Such proteins can be purified from blood or can be administered as a blood product or an enriched blood product. In one embodiment, highly purified factor VIII can be produced by adsorbing and eluting the factor from a blood product on a monoclonal antibody column. Alternatively, such naturally occurring proteins can be made recombinantly using nucleic acid molecules,

preferably naturally occurring nucleic acid molecules. For example, in one embodiment, factor VIII proteins are made by expressing a nucleic acid molecule encoding factor VIII in a cell, using techniques known in the art, such that factor VIII protein is produced. The nucleotide sequence (and the corresponding amino acid sequence) of human factor VIII is known in the art. (See e.g., Toole et al. Nature 1984. 312:5992; or GenBank Accession Nos. X01179; K01740).

In another embodiment, the agent which promotes hemostasis is a non-naturally occurring Factor VIII, e.g., mutant form of factor VIII which retains the therapeutic function, e.g., hemostasis promoting activity, of factor VIII. For example, DNA sequences capable of hybridizing to DNA encoding human factor VIII under conditions that avoid hybridization to non-factor VIII genes, (e.g., under conditions equivalent to 65°C in 5 X SSC (1 X SSC = 150 mM NaCl/ 0.15 M Na citrate)) or homologous DNA sequences which retain sequence identity over regions of the nucleic acid molecule which encode protein domains which are important in factor VIII function can be used to produce factor VIII proteins within the scope of the invention. As examples, the contents of U.S. Patents 5,744,446; 5,663,060; 5,583,209; 5,661,008; 5,422,260; and 5,707,832 are expressly incorporated herein by reference.

In another embodiment, an agent which promotes hemostasis is a factor VIII protein in which at least one domain (e.g., a nonessential domain) of the protein has been deleted. For example, in one embodiment, a factor VIII protein is a modified factor VIII protein in which one or more amino acids have been deleted or substituted between the 90 Kd and 69 Kd cleavage sites with respect to native factor VIII, as described in greater detail in United States Patent 4,868,112, the contents of which are incorporated herein by reference.

In another embodiment, the agent which promotes hemostasis is a factor VIII analog containing a deletion(s) of one or more amino acids between the 50/40 cleavage site and the 73 kD cleavage site which may be produced by methods analogous to those disclosed in United States Patent 4,868,112, the contents of which are hereby incorporated by this reference. In a preferred embodiment, a factor VIII analog retains part or all of the acidic amino acid region between the 80 kD and the 73 kD cleavage sites. In other embodiments part or all of this region is replaced with the corresponding acidic region immediately adjacent to the 50/40 cleavage site. In still other

embodiments, factor VIII proteins are analogs (with or without deletions as mentioned above) such as are disclosed in International Application PCT/US87/01299 (the contents of which are incorporated herein by reference), e.g. wherein one or more of the cleavage sites spanning arginine residues at positions 226, 336, 562, 740, 776, 1313, 1648 or 1721 have been rendered resistant to proteolytic cleavage, e.g., by replacement of one or more amino acids with different amino acids by mutagenesis of the cDNA using techniques known in the art, e.g., standard site directed mutagenesis.

Agents which promote hemostasis also include hybrid factor VIII proteins which include a portion of a human factor VIII protein and a portion of a non-human factor VIII protein from another species (e.g., porcine factor VIII). Such hybrid proteins can be made using techniques which are known in the art, e.g., as shown in U.S. Patents 5,744,446; 5,663,060; and 5,583,209.

The contents of U.S. Patents 5,693,499; 5,681,746; 5,663,060; 5,583,209; 5,563,045; 5,460,951; and 5,455,031 are also expressly incorporated herein by this reference.

In another embodiment, the agent which promotes hemostasis is factor IX. As used herein, the term "factor IX" includes, but is not limited to, factor IX isolated from plasma, transformed cell lines, and recombinantly produced factor IX isolated from host cell culture medium. Factor IX can be purified from blood or can be administered as a blood product or an enriched blood product. In one embodiment, highly purified factor IX can be produced by adsorbing and eluting the factor from a blood product on a monoclonal antibody column. Exemplary methods of purification are also disclosed in U.S. Patents 5,639,857; 5,457,181, and 5,286,849. Alternatively, such naturally occurring proteins can be made recombinantly using nucleic acid molecules, preferably naturally occurring nucleic acid molecules. For example, in certain embodiments, factor IX proteins are made by expressing a nucleic acid molecule encoding factor IX in a cell, using techniques known in the art, such that Factor IX protein is produced. Exemplary genetic constructs for expressing factor IX can be found in U.S. Patents 5,650,503 and 4,994,371.

The nucleotide sequence and amino acid sequence of factor IX is known in the art. (See, e.g., Yoshitake et al. 1985. *Biochemistry* 24:3726 or GenBank Accession Nos. K02402; A07407; A01819; or X54500).

In other embodiments, factor IX includes, for example, the proteins described in United States Patents 4,994,371; 5,171,569; 5,679,639; 5,621,039; and 5,714,583, the entire disclosures of which are each incorporated herein by reference.

In addition to naturally occurring forms of factor IX, the term factor IX also includes non-naturally occurring forms, e.g., mutant forms of factor IX which retain the therapeutic, e.g., hemostasis promoting properties of factor IX. For example, DNA sequences capable of hybridizing to DNA encoding human factor IX under conditions that avoid hybridization to non-factor IX genes, (e.g., under conditions equivalent to 65°C in 5 X SSC (1 X SSC = 150 mM NaCl/ 0.15 M Na citrate)). In addition, DNA sequences which retain sequence identity over regions of the nucleic acid molecule which encode protein domains which are important in factor IX function can be used to produce factor IX proteins within the scope of the invention.

Factor VIII or factor IX proteins can also be purchased commercially. For example, concentrated forms of factor VIII are available, e.g., Immunate® (Immuno), Beriate® (Behring); monoclonal antibody purified forms of factor VIII are available e.g., Octanativ-M® (Pharmacia), Hemofil M® (Baxter), and Monoclate-P® (Armour); and recombinant forms of factor VIII are also available, e.g., Recombinate® (Baxter) and Kogenate® (Bayer). A recombinant B-domain-deleted form of factor VIII, r-VIII SQ® (Pharmacia and Upjohn, Stockholm) is also available. Factor IX can be purchased, e.g., as Nanotiv® (Kabi Pharmacia) or Immunine® (Immuno); monoclonal antibody purified factor IX is also available as Mononine® (Armour). Recombinant factor IX is also available, e.g., as BeneFIX® (Genetics Institute).

VWF is a large multimeric plasma protein, composed of single glycoprotein subunits. The subunits of VWF are linked together by disulfide bonds. In plasma VWF circulates as multimers, ranging from dimers to multimers of more than 50 subunits. Dimers consist of two subunits joined, probably at their C-termini, by flexible "rod-shaped" domains and are presumed to be the protomers in multimerization. The protomers are linked through large, probably N-terminal, globular domains to form multimers. VWF appears to be produced as a 260 kD glycosylated precursor that is subsequently processed and sulfated. After dimerization and multimerization and proteolytic cleavage, the mature protein is about 225kD. VWF has been produced recombinantly. The nucleotide and amino acid sequence of VWF is known in the art.

(See e.g., Sadler et al. 1986. *Cold Spring Harbor Symposium in Qunatitative Biology* 51:515 or GenBank Accession Nos. L15333 or K03028). The contents of EP 0197592 B1 are incorporated herein by reference.

In addition to naturally occurring forms of factor VWF, the term "factor VWF also includes non-naturally occurring forms, e.g., mutant forms of factor VWF which retain the therapeutic properties, e.g., hemostasis promoting properties of factor VWF. For example, DNA sequences capable of hybridizing to DNA encoding human factor VWF under conditions that avoid hybridization to non-factor VWF genes, (e.g., under conditions equivalent to 65°C in 5 X SSC (1 X SSC = 150 mM NaCl/ 0.15 M Na citrate)). In addition, DNA sequences which retain sequence identity over regions of the nucleic acid molecule which encode protein domains which are important in factor VWF function can be used to produce factor VWF proteins within the scope of the invention.

In one embodiment, the agents which promote hemostasis are mammalian in origin. In a preferred embodiment, the agents which promote hemostasis are porcine in origin. In yet another more preferred embodiment, the agents for which promote hemostasis are human in origin. In another embodiment the agent which promotes hemostasis are hybrid molecules.

III. Immunomodulatory Agents

In one embodiment, an agent which inhibits a costimulatory signal in a T cell is a naturally occurring form of a costimulatory molecule. Naturally occurring forms of costimulatory molecules can be purified from cells or can be recombinantly produced using techniques known in the art. For example, costimulatory proteins can be made by expressing a nucleic acid molecule encoding a costimulatory molecule in a cell such that a costimulatory molecule is produced. Nucleotide sequences of costimulatory molecules are known in the art and can be found in the literature or on a database such as GenBank. See, for example, B7-2 (Freeman et al. 1993 *Science*. 262:909 or GenBank Accession numbers P42081 or A48754); B7-1 (Freeman et al. *J. Exp. Med.* 1991. 174:625 or GenBank Accession numbers P33681 or A45803; CTLA4 (See e.g., Ginsberg et al. 1985. *Science*. 228:1401; or GenBank Accession numbers P16410 or 291929); and CD28 (Aruffo and Seed. *Proc Natl. Acad. Sci.* 84:8573 or GenBank Accession number

180091), ICOS (Hutloff et al. 1999. *Nature.* 397:263; WO 98/38216), and related sequences.

In addition to naturally occurring forms of costimulatory molecules, the term "costimulatory molecule" also includes non-naturally occurring forms, e.g., mutant forms of costimulatory molecules which retain the function of a costimulatory molecule, e.g., the ability to bind to cognate counter receptor. For example, DNA sequences capable of hybridizing to DNA encoding a B7 molecule, a CTLA4 molecule, a CD28, or an ICOS molecule under conditions that avoid hybridization to non-costimulatory molecule genes, (e.g., under conditions equivalent to 65°C in 5 X SSC (1 X SSC = 150 mM NaCl/ 0.15 M Na citrate)) are costimulatory molecules within the scope of the invention. Alternatively, DNA sequences which retain sequence identity over regions of the nucleic acid molecule which encode protein domains which are important in costimulatory molecule function, e.g., binding to other costimultory molecules, can be used to produce costimulatory proteins which can be used as agents which inhibit a costimulatory signal in a T cell. Preferably, nonnaturally occurring costimulatory molecules have significant (e.g., greater than 70%, preferably greater than 80%, and more preferably greater than 90-95%) amino acid identity with a naturally occurring amino acid sequence of a costimulatory molecule extracellular domain.

To determine amino acid residues of a costimulatory molecule which are likely to be important in the binding of a costimulatory molecule to its counter receptor, amino acid sequences comprising the extracellular domains of costimulatory molecules of different species, e.g., mouse and human, can be aligned and conserved (e.g., identical) residues noted. This can be done, for example, using any standard alignment program, such as MegAlign (DNA STAR). Such conserved or identical residues are likely to be necessary for proper binding of costimulatory molecules to their receptors and are, thus, not likely to be amenable to alteration.

Specific residues of costimultory molecules which are important in binding have also been determined. For example, the portion of CD28 which is critical for interaction with B7-1 and B7-2 has been determined using site directed mutagenesis, CD28 monoclonal antibody epitope mapping, receptor based adhesion assays, and direct binding of Ig-fusion proteins to cell surface receptors. A stretch of proline rich sequence in CD28, MYPPPY, has been found to be critical to the function of that protein (Truneh

et al. 1996. *Mol. Immunol.* 33:321). Likewise, the regions of the B7-1 molecule which are important in mediating the functional interaction with CD28 and CTLA4 have been identified by mutation. Two hydrophobic residues in the V-like domain of B7-1, including the Y87 residue, which is conserved in all B7-1 and B7-2 molecules cloned from various species, were found to be critical (Fargeas et al. 1995. *J. Exp. Med.* 182:667). Using these, or similar, techniques amino acid residues of the extracellular domains of costimulatory molecules which are critical and, therefore, not amenable to alteration can be determined.

Costimulatory molecules can be expressed in soluble form or used as immunogens to make antibodies. Such soluble costimulatory molecules or antibodies are useful as agents which inhibit a costimulatory signal in a T cell as described in further detail herein.

A. Agents Which Act Extracellularly To Inhibit A Costimulatory Signal In A T Cell

1. Soluble forms of costimulatory molecules

In one embodiment, the agent which blocks a costimulatory signal in a T cell is a soluble form of a T cell costimulatory molecule (e.g., CTLA4, CD28, and/or ICOS) which is capable of blocking the transduction of a costimulatory signal in a T cell.

In one embodiment, the agent which blocks a costimulatory signal in a T cell is a soluble form of CTLA4. DNA sequences encoding the human and murine CTLA4 protein are known in the art, see e.g., Dariavich, et al. (1988) *Eur. J. Immunol.* 18(12), 1901-1905; Brunet, J.F., et al. (1987) *supra*; Brunet, J.F. et al. (1988) *Immunol. Rev.* 103:21-36; and Freeman, G.J., et al. (1992) *J. Immunol.* 149, 3795-3801. In certain embodiments, the soluble CTLA4 protein comprises the entire CTLA4 protein. In preferred embodiments, a soluble CTLA4 protein comprises the extracellular domain of a CTLA4 protein. For example, a soluble, recombinant form of the extracellular domain of CTLA4 has been expressed in yeast (Gerstmayer et al. 1997. *FEBS Lett.* 407:63). In other embodiments, the soluble CTLA4 proteins comprise at least a portion of the extracellular domain of CTLA4 protein which retains the ability to bind to B7-1 and/or B7-2.

In one embodiment the soluble CTLA4 protein or portion thereof is a fusion protein comprising at least a portion of CTLA4 which binds to B7-1 and/or B7-2 and at least a portion of a second non-CTLA4 protein. In preferred embodiments, the CTLA4 fusion protein comprises a CTLA4 extracellular domain which is fused at the amino terminus to a signal peptide, e.g., from oncostatin M (see e.g., WO93/00431).

In a particularly preferred embodiment, a soluble form of CTLA4 is a fusion protein comprising the extracellular domain of CTLA4 fused to a portion of an immunoglobulin molecule. Such a fusion protein, CTLA4Ig, can be made using methods known in the art (see e.g., Linsley 1994. *Perspectives in Drug Discovery and Design* 2:221; Linsley WO 93/00431 and U.S. Patent 5,770,197).

In one embodiment, the agent which blocks a costimulatory signal in a T cell is a soluble form an antigen presenting cell costimulatory molecule (e.g., a B7 family molecule, such as B7-1, B7-2 and/or an ICOS ligand. For example, in one embodiment, a soluble form of a costimulatory molecule includes a soluble form of B7-1 or a soluble form of B7-2 or a combination of a soluble form of B7-1 and a soluble form of B7-2. DNA sequences encoding B7 proteins are known in the art, see e.g., B7-2 (Freeman et al. 1993 *Science*. 262:909 or GenBank Accession numbers P42081 or A48754); B7-1 (Freeman et al. *J. Exp. Med.* 1991. 174:625 or GenBank Accession numbers P33681 or A45803. In certain embodiments, the soluble B7 protein comprises an entire B7 protein. In preferred embodiments, a soluble B7 protein comprises the extracellular domain of a B7 protein. For example, a soluble, recombinant form of the extracellular domain of CTLA4 has been expressed in yeast (Gerstmayer et al. 1997. *FEBS Lett.* 407:63). In other embodiments, the soluble B7 proteins comprise at least a portion of the extracellular domain of B7 protein which retains the ability to bind to CTLA4 and/or CD28.

In one embodiment the soluble B7 protein or portion thereof is a fusion protein comprising at least a portion of B7 which binds to CD28 and/or CTLA4 and at least a portion of a second non-B7 protein. In preferred embodiments, the B7 fusion protein comprises a B7 extracellular domain which is fused at the amino terminus to a signal peptide, e.g., from oncostatin M (see e.g., WO93/00431).

In a particularly more preferred embodiment, a soluble form of B7 is a fusion protein comprising the extracellular domain of B7 fused to a portion of an immunoglobulin molecule. Such a fusion protein, a B7Ig, can be made using methods known in the art (see e.g., Linsley 1994. *Perspectives in Drug Discovery and Design* 2:221; Linsley WO 93/00431, U.S. Patent 5,770,197, and U.S. Patent 5,580,756).

2. Antibodies which bind to costimulatory molecules

In certain embodiments, the agent which blocks a costimulatory signal in a T cell is an antibody which binds to a costimulatory molecule. In making antibodies which bind to costimulatory molecules, a costimulatory protein, a portion of a costimulatory protein, (e.g., a peptide derived from a costimulatory protein), or fusion protein which includes all or a portion of an amino acid sequence of a costimulatory molecule can be used to generate anti-protein and/or anti-peptide polyclonal antisera or monoclonal antibodies using standard methods. The term "antibody" as used herein is meant to include whole antibodies as well as fragments thereof. Fragments of antibodies (e.g., Fab' fragments, $F(ab')_2$ fragments, or single chain antibodies) can be made using methods well known in the art. The term antibody also includes chimeric and humanized antibodies.

For example, a mammal, (e.g., a mouse, hamster, or rabbit) can be immunized with an immunogenic form of the costimulatory protein or peptide which elicits an antibody response in the mammal. The immunogen can be, for example, a recombinant costimulatory molecule protein, or fragment thereof, a synthetic peptide fragment or a cell that expresses a costimulatory molecule on its surface. The cell can be, for example, an antigen presenting cell, or a T cell, or a cell transfected with a nucleic acid encoding a costimulatory molecule such that the costimulatory molecule is expressed on the cell surface. Host cells transfected to express peptides can be any procaryotic or eucaryotic cell. For example, a peptide having costimulatory molecule activity can be expressed in bacterial cells such as *E. coli,* insect cells (baculovirus), yeast, or mammalian cells such as Chinese hamster ovary cells (CHO) and NS0 cells. Other suitable host cells and expression vectors may be found in Goeddel, (1990) *supra* or are known to those skilled in the art. Examples of vectors for expression in yeast *S. cerivisae* include pYepSec1 (Baldari. et al., (1987) *Embo J.* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell*

30:933-943), pJRY88 (Schultz et al., (1987) *Gene* 54:113-123), and pYES2 (Invitrogen Corporation, San Diego, CA). Baculovirus vectors available for expression of proteins in cultured insect cells (SF 9 cells) include the pAc series (Smith et al., (1983) *Mol. Cell Biol.* 3:2156-2165) and the pVL series (Lucklow, V.A., and Summers, M.D., (1989) *Virology* 170:31-39). Generally, COS cells (Gluzman, Y., (1981) *Cell* 23:175-182) are used in conjunction with such vectors as pCDM8 (Seed, B., (1987) *Nature* 329:840) for transient amplification/expression in mammalian cells, while CHO (dhfr- Chinese Hamster Ovary) cells are used with vectors such as pMT2PC (Kaufman et al. (1987), *EMBO J.* 6:187-195) for stable amplification/expression in mammalian cells. A preferred cell line for production of recombinant protein is the NS0 myeloma cell line available from the ECACC (catalog #85110503) and described in Galfre, G. and Milstein, C. ((1981) *Methods in Enzymology* 73(13):3-46; and *Preparation of Monoclonal Antibodies: Strategies and Procedures*, Academic Press, N.Y., N.Y). Vector DNA can be introduced into mammalian cells via conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofectin, or electroporation. Suitable methods for transforming host cells can be found in Sambrook et al. (*Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory textbooks. When used in mammalian cells, the expression vector's control functions are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and most frequently, Simian Virus 40.

Peptides having an activity of a costimulatory molecule expressed in mammalian cells or otherwise can be purified according to standard procedures of the art, including ammonium sulfate precipitation, fractionation column chromatography (e.g. ion exchange, gel filtration, electrophoresis, affinity chromatography, etc.) and ultimately, crystallization (see generally, "Enzyme Purification and Related Techniques", *Methods in Enzymology*, 22:233-577 (1971)).

It will be appreciated by those skilled in the art that it is within their skill to generate antibodies to human costimulatory molecules by following standard techniques. Antibodies may either be polyclonal or monoclonal antibodies, or antigen binding fragments of such antibodies. Of particular significance for use in therapeutic applications are antibodies that inhibit binding of a costimulatory molecule with its

natural ligand(s) on the surface of immune cells, thereby inhibiting costimulation of the immune cell. Preferred anti-costimulatory molecule antibodies are those capable of inhibiting or downregulating T cell mediated immune responses by binding B7-2 or B7-1 on the surface of B lymphocytes and preventing interaction with CTLA4 and/or CD28. Other preferred anti-costimulatory molecule antibodies are those which, in combination with a second antibody which binds to another costimulatory molecule, results in increased inhibition of costimulation of a T cell when compared to the first antibody alone, e.g., a combination of anti-B7-1 and anti-B7-2 antibodies.

A. The Immunogen. The term "immunogen" is used herein to describe a composition containing a peptide having an activity of a costimulatory molecule as an active ingredient used for the preparation of antibodies against a costimulatory molecule. When a peptide having a costimulatory molecule activity is used to induce antibodies it is to be understood that the peptide can be used alone, or linked to a carrier as a conjugate, or as a peptide polymer.

To generate suitable anti-costimulatory molecule antibodies, the immunogen should contain an effective, immunogenic amount of a peptide having a costimulatory molecule activity, typically as a conjugate linked to a carrier. The effective amount of peptide per unit dose depends, among other things, on the species of animal inoculated, the body weight of the animal and the chosen immunization regimen as is well known in the art. The immunogen preparation will typically contain peptide concentrations of about 10 micrograms to about 500 milligrams per immunization dose, preferably about 50 micrograms to about 50 milligrams per dose. An immunization preparation can also include an adjuvant as part of the diluent. Adjuvants such as complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA) and alum are materials well known in the art, and are available commercially from several sources.

Those skilled in the art will appreciate that, instead of using naturally occurring forms of a costimulatory molecule for immunization, synthetic peptides can alternatively be employed towards which antibodies can be raised for use this invention. Both soluble and membrane bound costimulatory molecule or peptide fragments are suitable for use as an immunogen and can also be isolated by immunoaffinity purification as well. A purified form of a costimulatory molecule protein, such as may be isolated as described above or as known in the art, can itself be directly used as an immunogen, or

alternatively, can be linked to a suitable carrier protein by conventional techniques, including by chemical coupling means as well as by genetic engineering using a cloned gene of the a costimulatory molecule.

The peptide or protein chosen for immunization can be modified to increase its immunogenicity. For example, techniques for conferring immunogenicity on a peptide include conjugation to carriers or other techniques well known in the art. Any peptide chosen for immunization can also be synthesized. In certain embodiments, such peptides can be synthesized as branched polypeptides, to enhance immune responses, as is known in the art (see, e.g., Peptides. Edited by Bernd Gutte Academic Press 1995. pp. 456-493).

The purified costimulatory molecule protein can also be covalently or noncovalently modified with non-proteinaceous materials such as lipids or carbohydrates to enhance immunogenecity or solubility. Alternatively, a purified costimulatory molecule protein can be coupled with or incorporated into a viral particle, a replicating virus, or other microorganism in order to enhance immunogenicity. The costimulatory molecule protein may be, for example, chemically attached to the viral particle or microorganism or an immunogenic portion thereof.

In an illustrative embodiment, a purified costimulatory molecule protein, or a peptide fragment having a costimulatory molecule activity (e.g., produced by limited proteolysis or recombinant DNA techniques) is conjugated to a carrier which is immunogenic in animals. Preferred carriers include proteins such as albumin, serum proteins (e.g., globulins and lipoproteins), and polyamino acids. Examples of useful proteins include bovine serum albumin, rabbit serum albumin, thyroglobulin, keyhole limpet hemocyanin, egg ovalbumin and bovine gamma-globulins. Synthetic polyamino acids such as polylysine or polyarginine are also useful carriers. With respect to the covalent attachment of a costimulatory molecule protein or peptide fragments to a suitable immunogenic carrier, there are a number of chemical cross-linking agents that are known to those skilled in the art. Preferred cross-linking agents are heterobifunctional cross-linkers, which can be used to link proteins in a stepwise manner. A wide variety of heterobifunctional cross-linkers are known in the art, including succinimidyl 4-(N-maleimidomethyl) cyclohexane- 1-carboxylate (SMCC), m-Maleimidobenzoyl-N- hydroxysuccinimide ester (MBS); N-succinimidyl (4-

iodoacetyl) aminobenzoate (SIAB), succinimidyl 4-(p-maleimidophenyl) butyrate (SMPB), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC); 4-succinimidyloxycarbonyl- a-methyl-a-(2-pyridyldithio)-tolune (SMPT), N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), succinimidyl 6-[3-(2-pyridyldithio) propionate] hexanoate (LC-SPDP).

In may also be desirable to simply immunize with whole cells which express a costimulatory molecule protein on their surface. Various cell lines can be used as immunogens to generate monoclonal antibodies to a costimulatory molecule antigen, including, but not limited to activated B cells. For example, splenic B cells can be obtained from a subject and activated with anti-immunoglobulin. Alternatively, a B cell line can be used, provided that a costimulatory molecule is expressed on the cell surface, such as the Raji cell line (B cell Burkett's lymphoma, see e.g., Freeman, G.J. et al. (1993) *Science* 262:909-911) or the JY B lymphoblastoid cell line (see e.g., Azuma, M. et al. (1993) *Nature* 366:76-79). Whole cells that can be used as immunogens to produce costimulatory molecule specific antibodies also include recombinant transfectants. For example, COS and CHO cells can be reconstituted by transfection with a costimulatory molecule cDNA, such as described by Knudson et al. (1993, *PNAS* 90:4003-4007); Travernor et al. (1993, *Immunogenitics* 37:474-477); Dougherty et al. (1991, *J Exp Med* 174:1-5); and Aruffo et al. (1990, *Cell* 61:1303-1313), to produce intact costimulatory molecule on the cell surface. These transfectant cells can then be used as immunogen to produce anti-costimulatory molecule antibodies of preselected specificity. Other examples of transfectant cells are known, particularly eukaryotic cells able to glycosylate the costimulatory molecule protein, but any procedure that works to express transfected costimulatory molecule genes on the cell surface could be used to produce the whole cell immunogen.

B. Polyclonal Anti-Costimulatory Molecule Antibodies.

Polycolonal antibodies to a purified costimulatory molecule protein or peptide having a costimulatory molecule activity can generally be raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of a costimulatory molecule immunogen, such as the extracellular domain of a costimulatory molecule protein, and an adjuvant. For example, as described above, it may be useful to conjugate a

costimulatory molecule (including fragments containing particular eptitope(s) of interest) to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin.

The route and schedule of the host animal or cultured antibody-producing cells therefrom can generally make use of established and conventional techniques for antibody stimulation and production. In an illustrative embodiment, animals are typically immunized against the immunogenic costimulatory molecule conjugates or derivatives by combining about 1μg to 1mg of conjugate with Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of conjugate in Freund's complete adjuvant (or other suitable adjuvant) by subcutaneous injection at multiple sites. Seven to 14 days later, the animals are bled and the serum is assayed for anti-costimulatory molecule titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same costimulatory molecule protein, but conjugated to a different protein and/or through a different cross-linking agent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum can be used to enhance the immune response.

Such mammal-produced populations of antibody molecules are referred to as "polyclonal" because the population comprises antibodies with differing immunospecificities and affinities for a costimulatory molecule. The antibody molecules are then collected from the mammal and isolated by well known techniques such as, for example, by using DEAE Sephadex to obtain the IgG fraction. To enhance the specificity of the antibody, the antibodies may be purified by immunoaffinity chromatography using solid phase-affixed immunogen. The antibody is contacted with the solid phase-affixed immunogen for a period of time sufficient for the immunogen to immunoreact with the antibody molecules to form a solid phase-affixed immunocomplex. The bound antibodies are separated from the complex by standard techniques.

C. Monoclonal Anti-Costimulatory Molecule Antibodies. The term "monoclonal antibody" or "monoclonal antibody composition", as used herein, refers to a population of antibody molecules that contain only one species of an antigen binding

site capable of immunoreacting with a particular epitope of a costimulatory molecule. A monoclonal antibody composition thus typically displays a single binding affinity for a particular costimulatory molecule protein with which it immunoreacts. Preferably, the monoclonal antibody used in the subject method is further characterized as immunoreacting with a costimulatory molecule derived from humans.

Monoclonal antibodies useful in the compositions and methods of the invention are directed to an epitope of a costimulatory molecule antigen, such that complex formation between the antibody and the costimulatory molecule antigen inhibits interaction of the costimulatory molecule with its natural ligand(s) on the surface of immune cells, thereby inhibiting costimulation of a T cell through the costimulatory molecule-ligand interaction. A monoclonal antibody to an epitope of a costimulatory molecule can be prepared by using a technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein (1975, *Nature* 256:495-497), and the more recent human B cell hybridoma technique (Kozbor et al. (1983) *Immunol Today* 4:72), EBV-hybridoma technique (Cole et al. (1985), Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96), and trioma techniques. Other methods which can effectively yield monoclonal antibodies useful in the present invention include phage display techniques (Marks et al. (1992) *J Biol Chem* 16007-16010).

In one embodiment, the antibody preparation applied in the subject method is a monoclonal antibody produced by a hybridoma cell line. Hybridoma fusion techniques were first introduced by Kohler and Milstein (Kohler et al. *Nature* (1975) 256:495-97; Brown et al. (1981) *J. Immunol* 127:539-46; Brown et al. (1980) *J Biol Chem* 255:4980-83; Yeh et al. (1976) *PNAS* 76:2927-31; and Yeh et al. (1982) *Int. J. Cancer* 29:269-75). Thus, the monoclonal antibody compositions of the present invention can be produced by the following method, which comprises the steps of:

(a) Immunizing an animal with a costimulatory molecule. The immunization is typically accomplished by administering a costimulatory molecule immunogen to an immunologically competent mammal in an immunologically effective amount, i.e., an amount sufficient to produce an immune response. Preferably, the mammal is a rodent such as a rabbit, rat or mouse. The mammal is then maintained for a time period

sufficient for the mammal to produce cells secreting antibody molecules that immunoreact with the costimulatory molecule immunogen. Such immunoreaction is detected by screening the antibody molecules so produced for immunoreactivity with a preparation of the immunogen protein. Optionally, it may be desired to screen the antibody molecules with a preparation of the protein in the form in which it is to be detected by the antibody molecules in an assay, e.g., a membrane-associated form of a costimulatory molecule. These screening methods are well known to those of skill in the art.

(b) A suspension of antibody-producing cells removed from each immunized mammal secreting the desired antibody is then prepared. After a sufficient time, the mouse is sacrificed and somatic antibody-producing lymphocytes are obtained. Antibody-producing cells may be derived from the lymph nodes, spleens and peripheral blood of primed animals. Spleen cells are preferred, and can be mechanically separated into individual cells in a physiologically tolerable medium using methods well known in the art. Mouse lymphocytes give a higher percentage of stable fusions with the mouse myelomas described below. Rat, rabbit and frog somatic cells can also be used. The spleen cell chromosomes encoding desired immunoglobulins are immortalized by fusing the spleen cells with myeloma cells, generally in the presence of a fusing agent such as polyethylene glycol (PEG). Any of a number of myeloma cell lines may be used as a fusion partner according to standard techniques; for example, the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from the American Type Culture Collection (ATCC), Rockville, Md.

The resulting cells, which include the desired hybridomas, are then grown in a selective medium, such as HAT medium, in which unfused parental myeloma or lymphocyte cells eventually die. Only the hybridoma cells survive and can be grown under limiting dilution conditions to obtain isolated clones. The supernatants of the hybridomas are screened for the presence of antibody of the desired specificity, e.g., by immunoassay techniques using the antigen that has been used for immunization. Positive clones can then be subcloned under limiting dilution conditions and the monoclonal antibody produced can be isolated. Various conventional methods exist for isolation and purification of the monoclonal antibodies so as to free them from other proteins and other contaminants. Commonly used methods for purifying monoclonal

antibodies include ammonium sulfate precipitation, ion exchange chromatography, and affinity chromatography (see, e.g., Zola et al. in Monoclonal Hybridoma Antibodies: Techniques And Applications, Hurell (ed.) pp. 51-52 (CRC Press 1982)). Hybridomas produced according to these methods can be propagated *in vitro* or *in vivo* (in ascites fluid) using techniques known in the art.

Generally, the individual cell line may be propagated *in vitro*, for example in laboratory culture vessels, and the culture medium containing high concentrations of a single specific monoclonal antibody can be harvested by decantation, filtration or centrifugation. Alternatively, the yield of monoclonal antibody can be enhanced by injecting a sample of the hybridoma into a histocompatible animal of the type used to provide the somatic and myeloma cells for the original fusion. Tumors secreting the specific monoclonal antibody produced by the fused cell hybrid develop in the injected animal. The body fluids of the animal, such as ascites fluid or serum, provide monoclonal antibodies in high concentrations. When human hybridomas or EBV-hybridomas are used, it is necessary to avoid rejection of the xenograft injected into animals such as mice. Immunodeficient or nude mice may be used or the hybridoma may be passaged first into irradiated nude mice as a solid subcutaneous tumor, cultured *in vitro* and then injected intraperitoneally into pristane primed, irradiated nude mice which develop ascites tumors secreting large amounts of specific human monoclonal antibodies.

Media and animals useful for the preparation of these compositions are both well known in the art and commercially available and include synthetic culture media, inbred mice and the like. An exemplary synthetic medium is Dulbecco's minimal essential medium (DMEM; Dulbecco et al. (1959) *Virol* 8:396) supplemented with 4.5 gm/l glucose, 20 mM glutamine, and 20% fetal caf serum. An exemplary inbred mouse strain is the Balb/c.

D. Humanized Anti-Costimulatory Molecule Antibodies. When antibodies produced in non-human subjects are used therapeutically in humans, they are recognized to varying degrees as foreign and an immune response may be generated in the patient. One approach for minimizing or eliminating this problem, which is preferable to general immunosuppression, is to produce chimeric antibody derivatives,

i.e., antibody molecules that combine a non-human animal variable region and a human constant region. Such antibodies are the equivalents of the monoclonal and polyclonal antibodies described above, but may be less immunogenic when administered to humans, and therefore more likely to be tolerated by the patient.

Chimeric mouse-human monoclonal antibodies (i.e., chimeric antibodies) reactive with a costimulatory molecule can be produced, for example, by techniques recently developed for the production of chimeric antibodies. Humanized antibodies may be produced, for instance, by replacing an immunogenic portion of an antibody with a corresponding, but non-immunogenic portion. Accordingly, genes encoding the constant regions of the murine (or other species) anti-costimulatory molecule antibody molecule are substituted with genes encoding human constant regions. (Robinson et al., International Patent Publication PCT/US86/02269; Akira, et al., European Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison et al., European Patent Application 173,494; Neuberger et al., PCT Application WO 86/01533; Cabilly et al., European Patent Application 125,023; Better et al. (1988 *Science* 240:1041-1043); Liu et al. (1987) *PNAS* 84:3439-3443; Liu et al. (1987) *J. Immunol.* 139:3521-3526; Sun et al. (1987 *PNAS* 84:214-218; Nishimura et al. (1987) *Canc. Res.* 47:999-1005; Wood et al. (1985) *Nature* 314:446-449; and Shaw et al. (1988) *J. Natl Cancer Inst.* 80:1553-1559). General reviews of "humanized" chimeric antibodies are provided by Morrison, S. L. (1985) *Science* 229:1202-1207 and by Oi et al. (1986) *BioTechniques* 4:214. Those methods include isolating, manipulating, and expressing the nucleic acid sequences that encode all or part of an immunoglobulin variable region from at least one of a heavy or light chain. Sources of such nucleic acid are well known to those skilled in the art and, for example, may be obtained from an anti-costimulatory molecule antibody producing hybridoma. The chimeric cDNA can then be cloned into an appropriate expression vector.

Suitable "humanized" antibodies can be alternatively produced by CDR or CEA substitution (The Winter U.S. Patent 5,225,539; Jones et al. (1986) *Nature* 321:552-525; Verhoeyan et al. (1988) *Science* 239:1534; and Beidler et al. (1988) *J. Immunol.* 141:4053-4060).

E. Combinatorial Anti-Costimulatory Molecule Antibodies. Both monoclonal and polyclonal antibody compositions of the invention can also be produced by other methods well known to those skilled in the art of recombinant DNA technology. An alternative method, referred to as the "combinatorial antibody display" method, has been developed to identify and isolate antibody fragments having a particular antigen specificity, and can be utilized to produce monoclonal anti-costimulatory molecule antibodies, as well as a polyclonal anti-costimulatory molecule population (Sastry et al. (1989) *PNAS* 86:5728; Huse et al. (1989) *Science* 246:1275; and Orlandi et al. (1989) *PNAS* 86:3833). After immunizing an animal with a costimulatory molecule immunogen as described above, the antibody repertoire of the resulting B-cell pool is cloned. Methods are generally known for directly obtaining the DNA sequence of the variable regions of a diverse population of immunoglobulin molecules by using a mixture of oligomer primers and PCR. For instance, mixed oligonucleotide primers corresponding to the 5' leader (signal peptide) sequences and/or framework 1 (FR1) sequences, as well as primer to a conserved 3' constant region primer can be used for PCR amplification of the heavy and light chain variable regions from a number of murine antibodies (Larrick et al. (1991) Biotechniques 11: 152-156). A similar strategy can also been used to amplify human heavy and light chain variable regions from human antibodies (Larrick et al. (1991) *Methods: Companion to Methods in Enzymology* 2:106-110). The ability to clone human immunoglobulin V-genes takes on special significance in light of advancements in creating human antibody repertoires in transgenic animals (see, for example, Bruggeman et al. (1993) *Year Immunol* 7:33-40; Tuaillon et al. (1993) *PNAS* 90:3720-3724; Bruggeman et al. (1991) *Eur J Immunol* 21:1323-1326; and Wood et al. PCT publication WO 91/00906).

In an illustrative embodiment, RNA is isolated from activated B cells of, for example, peripheral blood cells, bone marrow, or spleen preparations, using standard protocols (e.g., U.S. Patent No. 4,683,202; Orlandi, et al. *PNAS* (1989) 86:3833-3837; Sastry et al., *PNAS* (1989) 86:5728-5732; and Huse et al. (1989) *Science* 246:1275-1281.) First-strand cDNA is synthesized using primers specific for the constant region of the heavy chain(s) and each of the κ and λ light chains, as well as primers for the signal sequence. Using variable region PCR primers, the variable regions of both heavy and light chains are amplified, each alone or in combinantion, and ligated into

appropriate vectors for further manipulation in generating the display packages. Oligonucleotide primers useful in amplification protocols may be unique or degenerate or incorporate inosine at degenerate positions. Restriction endonuclease recognition sequences may also be incorporated into the primers to allow for the cloning of the amplified fragment into a vector in a predetermined reading frame for expression.

The V-gene library cloned from the immunization-derived antibody repertoire can be expressed by a population of display packages, preferably derived from filamentous phage, to form an antibody display library. Ideally, the display package comprises a system that allows the sampling of very large variegated antibody display libraries, rapid sorting after each affinity separation round, and easy isolation of the antibody gene from purified display packages. In addition to commercially available kits for generating phage display libraries (e.g., the Pharmacia *Recombinant Phage Antibody System*, catalog no. 27-9400-01; and the Stratagene *SurfZAP*™ phage display kit, catalog no. 240612), examples of methods and reagents particularly amenable for use in generating a variegated anti-costimulatory molecule antibody display library can be found in, for example, the Ladner et al. U.S. Patent No. 5,223,409; the Kang et al. International Publication No. WO 92/18619; the Dower et al. International Publication No. WO 91/17271; the Winter et al. International Publication WO 92/20791; the Markland et al. International Publication No. WO 92/15679; the Breitling et al. International Publication WO 93/01288; the McCafferty et al. International Publication No. WO 92/01047; the Garrard et al. International Publication No. WO 92/09690; the Ladner et al. International Publication No. WO 90/02809; Fuchs et al. (1991) *Bio/Technology* 9:1370-1372; Hay et al. (1992) *Hum Antibod Hybridomas* 3:81-85; Huse et al. (1989) *Science* 246:1275-1281; Griffths et al. (1993) *EMBO J* 12:725-734; Hawkins et al. (1992) *J Mol Biol* 226:889-896; Clackson et al. (1991) *Nature* 352:624-628; Gram et al. (1992) *PNAS* 89:3576-3580; Garrad et al. (1991) *Bio/Technology* 9:1373-1377; Hoogenboom et al. (1991) *Nuc Acid Res* 19:4133-4137; and Barbas et al. (1991) *PNAS* 88:7978-7982.

In certain embodiments, the V region domains of heavy and light chains can be expressed on the same polypeptide, joined by a flexible linker to form a single-chain Fv fragment, and the scFV gene subsequently cloned into the desired expression vector or phage genome. As generally described in McCafferty et al., *Nature* (1990) 348:552-554,

complete V_H and V_L domains of an antibody, joined by a flexible $(Gly_4\text{-}Ser)_3$ linker can be used to produce a single chain antibody which can render the display package separable based on antigen affinity. Isolated scFV antibodies immunoreactive with a costimulatory molecule can subsequently be formulated into a pharmaceutical preparation for use in the subject method.

F. Hybridomas and Methods of Preparation. Hybridomas useful in the present invention are those characterized as having the capacity to produce a monoclonal antibody which will specifically immunoreact with a costimulatory molecule. As described below, the hybridoma cell producing anti-costimulatory molecule antibody can be directly implanted into the recipient animal in order to provide a constant source of antibody. The use of immuno-isolatory devices to encapsulate the hybridoma culture can prevent immunogenic response against the implanted cells, as well as prevent unchecked proliferation of the hybridoma cell in an immunocompromised host. A preferred hybridoma of the present invention is characterized as producing antibody molecules that specifically immunoreact with a costimulatory molecule expressed on the cell surfaces of activated human B cells.

Methods for generating hybridomas that produce, e.g., secrete, antibody molecules having a desired immunospecificity, i.e., having the ability to bind to a particular costimulatory molecule, and/or an identifiable epitope of a costimulatory molecule, are well known in the art. Particularly applicable is the hybridoma technology described by Niman et al. (1983) *PNAS* 80:4949-4953; and by Galfre et al. (1981) *Meth. Enzymol.* 73:3-46.

In another exemplary method, transgenic mice carrying human antibody repertoires can be immunized with a human costimultory molecule. Splenocytes from these immunized transgenic mice can then be used to create hybridomas that secrete human monoclonal antibodies specifically reactive with a human costimultory molecule (see, e.g., Wood et al. PCT publication WO 91/00906, Kucherlapati et al. PCT publication WO 91/10741; Lonberg et al. PCT publication WO 92/03918; Kay et al. PCT publication 92/03917; Lonberg, N. et al. (1994) *Nature* 368:856-859; Green, L.L. et al. (1994) *Nature Genet.* 7:13-21; Morrison, S.L. et al. (1994) *Proc. Natl. Acad. Sci. USA* 81:6851-6855; Bruggeman et al. (1993) *Year Immunol* 7:33-40; Tuaillon et al.

(1993) *PNAS* 90:3720-3724; and Bruggeman et al. (1991) *Eur J Immunol* 21:1323-1326).

The term "antibody" as used herein is intended to include fragments thereof which are also specifically reactive with a costimulatory molecule as described herein. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, $F(ab')_2$ fragments can be generated by treating antibody with pepsin. The resulting $F(ab')_2$ fragment can be treated to reduce disulfide bridges to produce Fab' fragments.

Antibodies made using these or other methods can be tested to determine whether they inhibit a costimulatory signal in a T cell using the methods described below.

In one embodiment the agent that inhibits a costimulatory signal in a T cell is an antibody which binds to both B7-1 and B7-2. In making such an antibody, for example, portions of the extracellular domain which are conserved between the two costimulatory molecules can be used as the immunogen. See, e.g., Metzler et al. 1997 *Nat Struct. Biol.* 4:527).

In one embodiment, the agent which inhibits a costimulatory signal in a T cell is an antibody which binds to B7-1. Such antibodies are known in the art or can be made as set forth above using a B7-1 molecule or a portion thereof as an immunogen and screened using the methods set forth above or other standard methods. Examples of B7-1 antibodies include those taught in U.S. Patent 5,747,034 and in McHugh et al. 1998. *Clin. Immunol. Immunopathol.* 87:50 or Rugtveit et al. 1997. *Clin Exp. Immunol.* 110:104.

In another embodiment, the agent which inhibits a costimulatory signal in a T cell is an antibody which binds to B7-2. Such antibodies are known in the art or can be made as set forth above using a B7-2 molecule or a portion thereof as an immunogen and screened using the methods set forth above or other standard methods. Examples of B7-2 antibodies include those taught in Rugtveit et al. 1997. *Clin Exp. Immunol.* 110:104.

In one embodiment, the agent which blocks a costimulatory signal in a T cell is a combination of an antibody which binds to B7-1 and an antibody which binds to B7-2.

In yet other embodiments, the agent which inhibits a costimulatory signal in a T cell is an antibody which binds to CD28, but does not transduce a costimulatory signal to a T cell (e.g., an Fab fragment of an anti CD28 antibody). Such antibodies are known in the art or can be made as set forth above using a CD28 molecule or a portion thereof as an immunogen and screened using the methods set forth above or other standard methods. Examples of known anti-CD28 antibodies include those taught by Darling et al. 1997. *Gene Ther.* 4:1350.

In preferred embodiments, Fab fragments of an antibody which binds to CD28 can be used. Such antibody fragments, which are unable to crosslink CD28 on the surface of a T cell, have been found to block T cell costimulation (Walunas et al. 1994. *Immunity* 1:405).

In yet other embodiments, the agent which inhibits a costimulatory signal in a T cell is an antibody which binds to CTLA4, which blocks a costimulatory signal in a T cell by delivering a negative signal to the T cell (i.e., which is a CTLA4 agonist). For example, crosslinking CTLA4 on the surface of a T cell has been shown to inhibit proliferation and IL-2 production (Krummel and Allison. 1996. *J. Exp. Med.* 183:2533). Such antibodies can be made as set forth above using a CTLA4 molecule or a portion thereof as an immunogen and screened using the methods set forth above or other standard methods. Exemplary antibodies are also taught e.g., in Vandenborre et al. 1998. *Am. J. Pathol.* 152:963.

In yet another embodiment, the agent which inhibits a costimulatory signal in a T cell is an antibody which binds to ICOS, which blocks a costimulatory signal in a T cell. Such antibodies can be made as set forth above using an ICOS molecule or a portion thereof as an immunogen and screened using the methods set forth above or other standard methods.

IV. Agents That Regulate The Expression Of Costimulatory Molecules

In another embodiment, an agent that inhibits a costimulatory signal in a T cell is an agent that interferes with the expression of a cositmulatory molecule. For example, interactions between CD40 on antigen presenting cells and CD40 ligand (CD40L) on T cells have been found to be important in sustaining, enhancing, or prolonging the expression of B7-1 or B7-2 on antigen presenting cells, resulting in enhanced

costimulation (Van Gool, et al. 1996. Immunol. Rev. 153:47; Klaus et al. 1994. J. Immunol. 152:5643).

In one embodiment, the agent which blocks the expression of a costimulatory molecule, thus blocking a costimulatory signal in a T cell is a soluble form of CD40 or CD40L. DNA sequences encoding these CD40 and CD40L are known in the art, see e.g., GenBank Accession Nos. Y10507 or Stamenlovic et al. 1988. *EMBO J.* 7:1053-1059 for CD40 or Gauchat et al. 1993. *FEBS* 315(3):259-266; Graf et al. 1992. *Eur. J. Immunol.* 22:3191-3194; Seyama 1996. *Hum. Genet.* 97:180-185 or GenBank Accession Nos. L07414, X67878, X96710 for CD40L.

In one embodiment, the agent which blocks the expression of a costimulatory molecule, thus blocking a costimulatory signal in a T cell is a soluble form of CD40 or CD40L. In one embodiment, the soluble CD40 or CD40L protein comprises the entire protein. In preferred embodiments, a soluble CD40 or CD40L protein comprises the extracellular domain of the protein. For example, a soluble, recombinant form of the extracellular domain of CD40 or CD40L protein or portion thereof can be made as a fusion protein comprising at least a portion of CD40 or CD40L such that the interaction between CD40 on an APC and CD40L on a T cell is interrupted and the delivery of a costimulatory signal to a T cell is inhibited. Such a soluble, recombinant form of a CD40 or CD40L protein comprises at least a portion of the molecule sufficient to bind to its counter receptor and at least a portion of a second non-CD40 or CD40L protein. In preferred embodiments, the CD40 or CD40L fusion protein comprises a CD40 or CD40 extracellular domain which is fused at the amino terminus to a signal peptide, e.g., from oncostatin M (see e.g., WO93/00431).

In a particularly preferred embodiment, a soluble form of CD40 or CD40L is a fusion protein comprising the extracellular domain of CD40 or CD40L fused to a portion of an immunoglobulin molecule (e.g., Chen et al. 1995. J. Immunol. 155:2833). Such a fusion protein, a CD40Ig or a CD40LIg, can be made using methods known in the art (see e.g., Linsley 1994. *Perspectives in Drug Discovery and Design* 2:221; Linsley WO 93/00431, U.S. Patent 5,770,197, and U.S. Patent 5,580,756).

In addition, antibodies to CD40 ligand have been found to synergize with agents which inhibit a costimulatory signal in a T cell to promote graft tolerance (Kirk et al. 1997. *Proc. Natl. Acad. Sci. USA* 94:8789; Larsen et al. 1996. *Nature* 381:434).

Therefore, in one embodiment, antibodies to CD40 or CD40L which bind to these molecules, but which do not induce the expression of costimulatory molecules, can be used as an agent which blocks a costimulatory signal in a T cell.

V. Agents That Act Intracellularly To Inhibit A Costimulatory Signal

In one embodiment, the agent which inhibits a costimulatory signal in a T cell is an agent which acts intracellularly to inhibit such a signal. Stimulation of a T cell through the CD28 surface receptor (i.e., a costimulatory signal) leads to the production of D-3 phosphoinositides in a T cell. Therefore, in one embodiment, the production of D-3 phosphoinositides can be inhibited in a T cell to inhibit a costimulatory signal to thereby inhibit a T cell response, as measured, for example, by T cell proliferation and/or cytokine production. The term "D-3 phosphoinositides" is intended to include derivatives of phosphatidylinositol that are phosphorylated at the D-3 position of the inositol ring and encompasses the compounds phosphatidylinositol(3)-monophosphate (PtdIns(3)P), phosphatidylinositol(3,4)-bisphosphate (PtdIns(3,4)P_2), and phosphatidylinositol(3,4,5)-trisphosphate (PtdIns(3,4,5)P_3).

D-3 phosphoinositides are generated intracellularly by the activity of a phosphatidyl-inositol 3-kinase (PI3K). PI3K is a heterodimer composed of an 85 kDa subunit that binds tyrosyl-phosphorylated proteins via its SH2 domains and a 110 kDa catalytic subunit. PI3K was first identified as a lipid kinase that phosphorylates the D-3 position of the inositol ring of phosphatidylinositol, PtdIns (4)P, and PtdIns(4,5)P2. Two recent studies have demonstrated that PI3K is in fact a dual-specificity kinase that possesses both lipid and serine kinase activities (Dhand, R. et al. (1994) *EMBO J.* 13:522 and Carpenter, C.L. et al. (1993) *Mol. Cell Biol.* 13:1657).

Accordingly, in one embodiment, the agent which inhibits a costimulatory signal in a T cell is an agent which inhibits the activity of a PI3K. A preferred agent which inhibits PI3K activity in a T cell is the fungal metabolite wortmannin, or derivatives or analogues thereof. Wortmannin is a potent PI3K inhibitor derived from *T. wortmannii* (Kyowa Hakko Kohyo Co. Ltd.) or from *P. fumiculosum* (Sigma). Wortmannin derivatives or analogues include compounds structurally related to wortmannin which retain the ability to inhibit PI3K and T cell responses. Examples of wortmannin derivatives and analogues are disclosed in Wiesinger, D. et al. (1974) *Experientia*

30:135-136; Closse, A. et al. (1981) *J. Med. Chem.* 24:1465-1471; and Baggiolini, M. et al. (1987) *Exp. Cell Res.* 169:408-418. Another inhibitor of PI3K activity that can be used is the bioflavenoid quercetin, or derivatives or analogues thereof. Quercetin derivatives or analogues include compounds structurally related to quercetin that retain the ability to inhibit PI3K and inhibit T cell responses. Examples of quercetin derivatives and analogues are disclosed in Vlahos, C.J. et al. (1994) *J. Biol. Chem.* 269:5241-5284. A preferred quercetin derivative which inhibits PI3K activity is LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one, Lilly Indianapolis, IN) (described in Vlahos et al. cited *supra*).

CD28 stimulation has also been shown to result in protein tyrosine phosphorylation in a T cell (see e.g., Vandenberghe, P. et al. (1992) *J. Exp. Med.* 175:951-960; Lu, Y. et al. (1992) *J. Immunol.* 149:24-29). Accordingly, in one embodiment, an agent which inhibits a costimulatory signal in a T cell inhibits tyrosine phosphorylation in the T cell. A preferred protein tyrosine kinase inhibitor is one which inhibits *src* protein tyrosine kinases. In one embodiment, the *src* protein tyrosine kinase inhibitor is herbimycin A, or a derivative or analogue thereof. Derivatives and analogues of herbimycin A include compounds which are structurally related to herbimycin A and retain the ability to inhibit the activity of protein tyrosine kinases. In another embodiment, the agent which inhibits protein tyrosine phosphorylation is a protein tyrosine phosphatase or an activator of a protein tyrosine phosphatase. By increasing the tyrosine phosphatase activity in a T cell, the net amount of protein tyrosine phosphorylation is decreased. The protein tyrosine phosphatase can be a cellular protein tyrosine phosphatase within the T cell, such CD45 or Hcph. The activity of a cell surface tyrosine phosphatase on a T cell can be activated by contacting the T cell with a molecule which binds to the phosphatse and stimulates its activity. For example, an antibody directed against CD45 can be used to stimulate tyrosine phosphatase activity in a T cell expressing CD45 on its surface. Accordingly, in one embodiment, the agent which inhibits protein tyrosine phosphorylation within the T cell is an anti-CD45 antibody, or a fragment thereof which retains the ability to stimulate the activity of CD45. Examples of antibody fragments include Fab and F(ab')2 fragments. Antibodies, or fragments thereof, can be provided in a stimulatory form, for example multimerized or immobilized etc.

In addition, CD28 ligation has been associated with increased phospholipase C activity (see e.g., Nunes, J. et al. (1993) *Biochem. J.* 293:835-842) and increased intracellular calcium levels (see e.g. Ledbetter, J.A. et al. (1990) *Blood* 75:1531-1539 and the Examples). Accordingly, an agent which acts intracellularly to inhibit a costimulatory signal in a T cell can act by inhibiting phospholipase C activity and/or inhibiting an increase in intracellular calcium levels. For example, the tyrosine kinase inhibitor herbimycin A also inhibits CD28-induced calcium flux in T cells.

Protein serine and serine-threonine kinases have also been shown to be involved in signal transduction pathways associated with CD28 (Siegel, J.N. et al. (1993) *J. Immunol.* 151:4116-4127; Pai, S.V. et al. (1994) *J. Immunol.* 24:2364; Parry et al. 1997. *Eur. J. Immunol.* 27:2495). Thus, in another embodiment of the invention, an agent which acts intracellularly to inhibit a costimulatory signal in a T cell inhibits serine or serine-threonine kinase activity.

VI. Other Agents That Block Costimulation Of T Cells

Other agents which block a costimulatory signal in a T cell can be identified using standard techniques. For example, such agents can be identified by their ability to inhibit T cell proliferation and/or cytokine production. For instance, a costimulation assay system can be used. In such a system, human $CD28^+$ T cells are isolated for example, by immunomagnetic bead depletion using monoclonal antibodies directed against B cells, natural killer cells and macrophages as previously described (Gimmi, C.D., et al. (1993) *Proc. Natl. Acad. Sci. USA* 90, 6586-6590). Antigen presenting cells, e.g., whole spleen cells, or purified B cells, or B7-1 or B7-2 transfected COS cells can be irradiated or treated with mitomycin-C (e.g., at 25μg/ml) for 1 hour, and then extensively washed to inhibit proliferation. 10^5 $CD28^+$ T cells can be incubated with, e.g., 10^5- 10^4 APCs, (e.g., COS cells transfected with a B7 molecule). In this exemplary assay, one population of the T cells receive a primary activation signal (e.g., a T cell receptor signal) alone; another population of T cells receive a costimulatory signal alone; yet another population of T cells receive both a primary activation signal and a costimulatory signal and yet another population of T cells receive both a primary activation signal and a costimulatory signal in the presence of the agent to be tested for its ability to block a costimulatory signal in a T cell. A primary activation signal can be

delivered, e.g., by a submitogenic dose of PMA (e.g., 1ng/ml), a submitogenic dose of mitogen, a suboptimal dose of antigen, or a submitogenic dose of anti-T cell receptor antibody. Signal 2 is delivered by antigen presenting cells bearing a B7 molecule. Potential blocking agents can be tested at a range of concentrations. For example, potential blocking antibodies can be used as hybridoma supernatants or as purified antibody (e.g., at about 10μg/ml). Proliferation of T cells can be measured by ^{3}H-thymidine (1μCi) incorporation for the last 12-18 hours of a 72 hour incubation. The delivery of a primary activation signal should result in some proliferation, but T cells receiving both the primary activation signal and costimulatory signal 2, signals should proliferate maximally. Blocking agents are identified by their ability to reduce the maximal, costimulatory signal induced proliferation.

In addition to, or as an alternative to measuring T cell proliferation, T cell cytokine production can be measured using techniques which are well known in the art. For example, IL-2 and IL-4 produced in the T cell cultures can be assayed in culture supernatants collected at 24-72 hours after initiation of the culture using a commercially available ELISA (R&D Systems, Minneapolis, MN and BioSource, Camarillo, CA). As set forth above, blocking agents can be identified by their ability to reduce the maximal, costimulatory signal induced cytokine production.

In the case of antibodies, any "blocking antibodies" identified using this or another assay can be further tested to determine the costimulatory molecule to which they bind, using techniques known in the art. For example, the ability of the blocking antibody to reduce the binding of a labeled antibody to a known ligand can be measured.

VII. Additional Agents For Downmodulating Immune Responses

In certain embodiments, the compositions and methods of the invention can comprise additional agents or the use of additional agents to enhance immunotolerance to an agent which promotes hemostasis. In one embodiment, an agent which promotes immunotolerence but does not act by inhibiting a costimulatory signal in a T cell can be added to the instant compositions or administered in the instant methods. For example, anti-CD40 ligand (e.g., the monoclonal antibody to human CD40 ligand, 5C8 (Kirk et al. 1997. *Proc. Natl. Acad. Sci. USA* 94:8789)) can be included in a composition. CD40 and its T cell-based ligand, CD40L (CD154) play an important role in up-regulating B7

and in establishing B cell activity (U.S. Patent 5,683,693, Yang et al. 1996. *Science* 273:1862; Grewal et al. 1996. *Science* 273:1864; Leterman et al. 1992. *J. Exp. Med.* 175:1091; Lederman et al. 1992. *J. Immunol.* 149:3817). Antibodies to CD40 ligand have been found to synergize with agents which inhibit a costimulatory signal in a T cell to promote graft tolerance (Kirk et al. 1997. *Proc. Natl. Acad. Sci. USA* 94:8789; Larsen et al. 1996. *Nature* 381:434). In another embodiment, an agent that acts intracellularly to promote immunotolerization, but which does not inhibit a costimulatory signal in a T cell can be used in the subject compositions. For example, in one embodiment, cyclosporine A (CSA), FK506, Rapamycin, or another agent which inhibits immune responses can be included in the instant compositions or administered as part of the instant methods. (See, e.g., Sigal et al. 1992. *Annv. Rev. Immunol.* 10:519, Ruhlmann et al. 1997or *Immunobiology.* 198:192; Shaw et al. 1996. *Clin. Chem.* 42:1316)

VIII. Methods of Using Compositions Which Comprise a Therapeutic Protein and an Immunotolerizing Agent

In one embodiment the subject compositions and/or agents described herein are administered to subjects who have a hemostatic disorder and have been previously treated with an agent that promotes hemostasis. In another embodiment, the subject compositions and/or agents described herein are administered to subjects who have not yet been treated with an agent that promotes hemostasis.

In yet another embodiment, the subject compositions and/or agents described herein are administered to a subject that has not yet developed an immune response to an agent which promotes hemostasis. In other embodiments, the subject compositions and/or agents are administered to subjects who have a preexisting immune response to an agent which promotes hemostasis. Whether a subject has a "preexisting immune response" can be determined by measuring the titer of antibodies in the subject that react with the agent that promotes hemostasis using techniques that are well known in the art. If such a subject has a measurable titer of such antibodies (e.g., a statistically significant titer) when compared with titers from control individuals, a subject can be said to have a preexisting immune response to an agent which promotes hemostasis. Alternatively, a cellular immune response to an agent that promotes hemostasis can be measured to

determine whether a subject has an ongoing immune response to an agent that promotes hemostasis. Such techniques are well known in the art.

In one embodiment, a first agent which promotes hemostasis and a second agent which inhibits or blocks a costimulatory signal in a T cell are used to treat a hemostatic disorder. In another embodiment, compositions comprising a combination of a first agent which promotes hemostasis and a second agent which inhibits a costimulatory signal in a T cell can be used to treat a hemostatic disorder.

Administration of the compositions and/or agents described herein can be in any pharmacological form that includes a therapeutically active amount of an agent and a pharmaceutically acceptable carrier. Administration of a therapeutically active amount of the subject agents and/or compositions is defined as an amount effective, at dosages and for periods of time necessary to achieve treatment of the hemostatic disorder in the case of an agent which promotes hemostasis, and to achieve immunotolerance to the agent which promotes hemostasis in the case of an agent which inhibits a costimulatory signal in a T cell. A therapeutically active amount of an agent or composition may vary according to factors such as the disease state, age, sex, and weight of the individual, and whether or not the individual has already developed an immune response to an agent which promotes hemostasis. Such an amount can be readily determined by one of ordinary skill in the art.

The optimal course of administration of the agents and/or compositions may also vary depending upon the subject to be treated. In certain embodiments a subject will require treatment with both agents at one time. In that instance, it will be desirable to administer an agent which promotes hemostasis and an agent which inhibits a costimulatory signal in a T cell simultaneously, for example, in the form of a composition comprising both agents.

In other embodiments it will be desirable to administer the agents separately, e.g., in order to promote stability of the agents, or to facilitate staggered administration of the agents. In one embodiment, staggered administration may be desirable to achieve optimal therapeutic effect of the agent which promotes hemostasis, while optimally inhibiting the immune response, preferably an antibody response, to the agent. For example, an agent which inhibits a costimulatory signal can be administered alone prior

to administration of an agent which promotes hemostasis, or can be administered alone for several days after administering an agent that promotes hemostasis.

In one embodiment, an agent which blocks a costimulatory signal in a T cell can be administered chronically, e.g., each time the agent which promotes hemostasis is administered. In another embodiment, an agent which blocks a costimulatory signal in a T cell is administered sporadically. For example, a subject may require treatment with an agent that promotes hemostasis on a regular basis, but may only require treatment with an agent that inhibits a costimulatory signal in a T cell periodically. For example, treatment with an agent which promotes hemostasis can be ongoing, while as few as one or two treatments of the subject with an agent which blocks a costimulatory signal in a T cell may be sufficient; further administration of an agent which blocks a costimulatory signal may not be required. In a preferred embodiment, an agent that blocks a costimulatory signal in a T cell is administered at appropriate intervals for at least about 6 months.

In one embodiment the subject agents or compositions are administered to patients if the patient is found to have preexisting antibodies. In another embodiment, the subject agents or compositions are administered to previously untreated patients, i.e., without preexisting antibodies. In still another embodiment, the subject agents or compositions are administered to patients that have been previously treated, but do not have antibody titers against an agent which promotes hemostasis.

A dosage regime may be adjusted to provide the optimum therapeutic response for each subject without undue experimentation. For example, antibody titers to an agent which promotes hemostasis can be measured to determine whether or not the subject is developing an immune response to the agent and the dosage regimen can be adjusted accordingly. For example, if antibody titers to an agent that promotes hemostasis increase, more doses of an agent which blocks a costimulatory signal in a T cell may be administered.

To administer the subject agents or compostions by other than parenteral administration, it may be necessary to coat them with, or co-administer them with, a material to prevent its inactivation. An agent or compostion of the present invention may be administered to an individual in an appropriate carrier or diluent, co-administered with enzyme inhibitors or in an appropriate carrier such as liposomes.

Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Enzyme inhibitors include pancreatic trypsin inhibitor, diisopropylfluorophosphate (DEP) and trasylol. Liposomes include water-in-oil-in-water emulsions as well as conventional liposomes (Strejan et al., (1984) *J. Neuroimmunol* 7:27).

The active agent or composition may also be administered parenterally or intraperitoneally. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases, the agent or composition must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, asorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating active composition or agent in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable

solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient (e.g., agent or comosition) plus any additional desired ingredient from a previously sterile-filtered solution thereof.

When the active agent or composition is suitably protected, as described above, the protein may be orally administered, for example, with an inert diluent or an assimilable edible carrier. As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the therapeutic compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active agent or composition for the treatment of individuals.

As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Supplementary agents can also be incorporated.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, *Genetics*; *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, J. *et al.* (Cold Spring Harbor

Laboratory Press (1989)); *Short Protocols in Molecular Biology*, 3rd Ed., ed. by Ausubel, F. *et al.* (Wiley, NY (1995)); *DNA Cloning*, Volumes I and II (D. N. Glover ed., 1985); *Oligonucleotide Synthesis* (M. J. Gait ed. (1984)); Mullis *et al.* U.S. Patent No: 4,683,195; *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. (1984)); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London (1987)); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds. (1986)); and Miller, J. *Experiments in Molecular Genetics* (Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1972)).

The contents of all references, pending patent applications and published patents, cited throughout this application are hereby expressly incorporated by reference.

The invention is further illustrated by the following examples, which should not be construed as further limiting.

EXAMPLES

In the Examples, a mouse model of hemophilia A was used to evaluate new methods for the prevention and treatment of inhibitor formation. Hemophilia A mice, generated by targeted disruption of exon 16 of Factor VIII gene, have no detectable Factor VIII activity in their plasma (Bi L, Nature Genetics 10:119, 1995) and are similar in this way to patients with severe hemophilia A. As expected, hemophilia A mice have in vivo signs of a coagulation pathway defect with fatal bleeding if tails are cut without use of hemostatic measures, and they develop subcutaneous and intramuscular bleeding after handling or temporary immobilization (Qian J, Borovok M, Bi L, Kazazian HH, Hoyer L. Thromb Haemost 81 :940, 1999; Evans GL et al, Proc Natl Acad Sci USA 95:5734, 1998).

Intravenous infusions of 0.2μg human factor VIII, a dose equivalent on a weight basis to that given to hemophilia A patients, resulted in minimal or no antibody response in these hemophilia A mice after a single injection, but repeat infusions led to high titer inhibitory anti-factor VIII (Qian J et al. Thromb Haemost 81 :940, 1999). In addition, a factor VIII-specific T cell proliferative response was detected three days after first exposure to human factor VIII, before antibodies was detected.

Example 1. Inhibition of the primary immune response to factor VIII

The experimental design for this example is shown in Figure 1. Three groups of mice were injected with factor VIII intravenously on day 0. One group of mice was also injected with CTLA4-Ig one day before and one day after the factor VIII injection. A second group of mice received the same CTLA4-Ig treatment (intraperitoneally) followed by daily factor VIII injections from day 2 to day 12. The third group did not receive any CTLA4-Ig. All mice then received two additional injections of factor VIII at days 23 and 44. Animals were bled at days 20, 37, 58, and 82. While mice that did not receive CTLA4Ig had high titers of antibody beginning as early as day 20, mice that received CTLA4Ig did not develop antibodies until day 82 (Figure 2).

Example 2. Inhibition of the secondary response to factor VIII

The experimental design for this example is shown in Figure 3. In this example mice were given multiple intravenous injections of factor VIII at two week intervals and then divided into two groups. The mice were re-injected with factor VIII at days 1, 20, and 37. One group of mice was injected with CTLA4-Ig on days -1 and day +1 relative to the day 1, 20, and 37 factor VIII injections. The animals that did not receive CTLA4Ig had high titers of anti-factor VIII antibodies, while the mice that received CTLA4Ig (with the exception of 1 mouse) did not develop a secondary immune response to factor VIII (Figure 4).

The following methods and materials were used in Examples 3-6.

Animals. The characteristics of the exon-16 (E-16) strain of hemophilic mice have been reported Bi L, et al. Nature Genetics 10:119, 1995; Bi L, et al. Blood 88:3446, 1996. Adult male and homozygous E-16 female mice, aged 10-20 weeks, were used for these studies. Blood samples were obtained by orbital venous plexus bleeding, and the serum was separated by centrifugation at 600 g for 3 minutes. The serum samples were stored at -20°C until assayed. To avoid severe bleeding and death of animals, ear tags were not used to identify the mice in some experiments. For this reason, Figure 5 does not indicate sequential data for individual mice.

Generation of E-16/B7-1 and B7-2 double knockout mice was accomplished by cross breeding of E-16 with B7-1 and B7-2 knockout mice (Borriello F, et al. 1997. Immunity 6:303). Homozygous E-16/B7-1 and E-16/B7-2 double knockout mice were identified by genotype determination (Bi L et al, Nature Genetics 10:119, 1995; Borriello F, et al. Immunity 6:303, 1997. Reduced factor VIII activity was verified using the Coatest chromogenic bioassay (Chromogenix, MoIndal, Sweden) (Bi L et al, Blood 88:3446, 1996). The factor VIII activity was less then 1% in both E-16/B7-1 and E-16/B7-2 deficient mice.

Antigens. Recombinant human factor VIII was obtained from the Hyland Division of Baxter Healthcare Corp. (Glendale, CA).

mCTLA-4Ig. A murine CTLA4-lg cDNA expression plasmid was prepared by ligation of the leader and extracellular domains of murine CTLA4 to the hinge, CH2 and CH3 domains of IgHg2a that had been mutated to remove effector functions as described in Streurer et al (Streurer, J Immunol 155:1165, 1995). The insert was cloned into the expression vector pED and stably transfected into CHO cells as previously described (Lollar P et al, J Clin Invest 93:2497, 1994). Concentrated conditioned media was loaded onto a rProtein A Sepharose Fast Flow chromatography column (Amersham Pharmacia Biotech, Piscataway, NJ). The column was washed with PBS pH 7.1 and the mCTLA4-lg eluted with 20 mM Citrate pH 3.0. The peak pool was neutralized with 1M Tris pH 8.0 to a final pH of 7.5 and formulated into PBS pH 7.1 using an Amicon stirred cell with a YM3O membrane. The mCTLA4-Ig was depyrogenated using a Poros PI (Perceptive Biosystems) chromatography column and the product eluted from the column in a linear NaCl gradient from O to I M NaCl in 25 mM Tris pH 7.5. The mCTLA-4-Ig was then formulated into PBS pH 7.1 using an Amicon stirred cell using a YM30 membrane.

Antibody Measurements. The anti-factor VIII titer was determined by ELISA (Qian J, et al. Inhibitor antibody development and cell response to human factor VIII in murine hemophilia A. Thromb Haemost 81 :940, 1999). The ELISA assays were carried out using microtiter wells coated with recombinant human factor VIII, 0.8μg/ml in 0.05

mol/ml carbonate-bicarbonate, pH 9. After mouse plasma samples were incubated in the wells at 4°C overnight and then washed, alkaline-phosphatase conjugated goat anti-mouse IgG (Southern Biotechnology Associates Inc., Birmingham, AL) was added for 2 hours at room temperature. After washing, P-nitrophenyl phosphate (Sigma, St. Louis, MO), 2mg/ml in 100mmol/L glycine, 1mmol/L $MgCl_2$, 2mmol/L $ZnCl_2$, pH 10.4, was then added and the absorbance read at 410 nm using an automated micro titer plate ELISA reader. The concentration of anti-factor VIII antibody was estimated from a standard curve obtained using a monoclonal murine IgG anti-human factor VIII antibody that binds to the A2 domain (Mab 413) (Lollar P et al, J Clin Invest 93:9497, 1994). The titer was calculated from points that fell on the linear portion of the assay standard curve.

Anti-factor VIII inhibitor titers in Bethesda Units (BU) were measured by the Bethesda Assay (Kasper CK. Thromb et Diath Haem 30:263, 1973).

T cell proliferation assays. The spleen was used as the source of T cells for proliferation assays. Spleen cells were then cultured (5×10^5/well) in 96 well flat bottom plates. Varying amounts of recombinant factor VIII were added to the culture medium consisting of complete RPMI-1640 containing 0.5% hemophilic mouse serum. 37 kBp of ^{3}H-thymidine/well (6.7Ci/mmol, ICN Pharmaceuticals Irvine, CA) was added after 72 hours of culture at 37°C. The cultures were harvested 16 hours later using a Matrix 9600 (Packard, Meriden, CT). Data are expressed as the mean for triplicate wells of the cpm incorporated into insoluble DNA.

Example 3. mCTLA4-Ig blocks the induction of an anti-factor VIII response.

Anti-factor VIII inhibitory antibodies were induced in control mice by repeated intravenous injections of 1μg recombinant human factor VIII at three week intervals. In this Example, four groups of hemophilia A mice were injected with recombinant human factor VIII on days 0, 23, 44 and 66 (1μg i.v. initially, and then 0.2μg for the 2nd, 3rd, and 4th injections). Mice in groups G-3 and G-4 were also injected intraperitoneally with 0.2μg factor VIII on days 2-12. Blood samples for anti-factor VIII assay were obtained on days 20, 37, 58 and 82. Control groups G-1 and G-3, open circles, were injected with only factor VIII. Groups G-2 and G-4, solid circles, were also injected with mCTLA4-Ig

(250μg, i.p.) on the day before and the day after the first factor VIII injection. The anti-factor VIII antibody concentration was determined by ELISA. Anti-factor VIII assay data points indicated as <0.16μg/ml were similar to those for plasma samples obtained from unimmunized hemophilia A mice. The results of this experiment are shown in Figure 5 (Note that Figure 5 repeats some of the data shown in Figure 2, but adds additional data).

Anti-factor VIII was detected in four of the five mice 20 days after the first injection, and all control mice developed high titer anti-factor VIII after receiving two to four injections. The mean inhibitor level after four injections was 1860 Bethesda units (BU). Anti-factor VIII antibody formation was markedly suppressed in mice injected intraperitoneally with 250μg of murine CTLA4-lg on the day before and the day after first factor VIII injection (Group G-2, Figure 5), even though there was no further mCTLA4-lg given with three subsequent factor VIII injections on days 23, 44 and 66. Anti-factor VIII was not detectable in any Group G-2 mice after the first or second factor VIII injection. Three weeks after the third injection of factor VIII, a weak immune response was detected in two of the six mice in the G-2 group.

To investigate if the limited duration of unresponsiveness is a result of the short half life of human factor VIII in these mice (4-5 hours in murine hemophilia A (Evans GL et al, Proc Natl Acad Sci USA 95:5734, 1998), control and mCTLA-4-Ig treated mice (Groups G-3 and G-4, Figure 5) were injected with 1μg factor VIII intravenously on day O followed by daily intraperitoneal injections of 1μg factor VIII on days 2-12. High titer anti-factor VIII was present on day 20 in the control mice (Group G-3): over 350μg/ml by ELISA and a mean inhibitor titer of 694 BU. In contrast, the Group G-4 mice that were injected with mCTLA4-Ig on the day before and the day after the first exposure to factor VIII had no detectable anti-factor VIII on day 20. The delayed anti-factor VIII antibody response after three additional factor VIII injections was the same in these mice as that in the Group G-2 animals. Thus, the limited persistence of factor VIII in the plasma after CTLA4-Ig injection was not the reason for a limited duration of unresponsiveness in CTLA4-Ig treated mice.

Example 4. Effects of Repeated Administration of CTLA4-Ig

Because a delayed anti-factor VIII response was detected after repeated factor VIII infusions when mCTLA4-Ig was given only at the time of the first factor VIII exposure, it was determined if mCTLA4-Ig might prevent anti-factor VIII development if given with each factor VIII infusion. In that experiment (Figure 6), hemophilia A mice were simultaneously infused with both factor VIII and mCTLA4-Ig six times at three week intervals. Hemophilia A mice were injected intravenously with both 1μg factor VIII and 250μg mCTLA4-Ig at 3 week intervals (solid circles) or with factor VIII alone after the first injection which contained both factor VIII and mCTLA4-Ig (open circles). Serum samples for anti-factor VIII assay were obtained 4 weeks after the 6th factor VIII injection. There was no detectable anti-factor VIII in any of ten mice treated in this way when they were tested four weeks after the sixth factor VIII injection. In contrast, high titer anti-factor VIII was present in serum from mice that had received only one mCTLA4-Ig injection (at the time of the first exposure to factor VIII) followed by five injections of factor VIII alone.

These mCTLA4-Ig treated mice were then tested to determine if they would have an immune response after additional factor VIII injections in the absence of mCTLA4-Ig. After two intravenous injections at three week intervals, none of five mice developed anti-factor VIII, while low level anti-factor VIII was detected in two of four control mice not previously exposed to either factor VIII or mCTLA4-Ig (Figure 7). In this experiment, hemophilia A mice treated as described for Figure 6 with six injections of both factor VIII and mCTLA4-Ig were subsequently given six intravenous injections of 0.2μg of factor VIII at 3 week intervals without additional mCTLA4-Ig (closed circles). Control mice with no prior factor VIII exposure were immunized in parallel (open circles). Serum samples for anti-factor VIII assay were obtained 3 weeks after the 2nd and 6th injections. After six injections of factor VIII alone, the mean factor VIII titer was 93μg/ml for mice that had previously received both factor VIII and mCTLA4-Ig, while the mean titer was 155μg/ml for the control mice. These data document the limits of antigen-specific immune suppression that follow from repeated co-administration of mCTLA4-Ig with factor VIII.

Example 5. mCTLA4-Ig suppresses the secondary immune response to factor VIII.

To determine if mCTLA4-lg modifies a secondary immune response to factor VIII, mCTLA4-Ig was injected at the same time that factor VIII was given to hemophilia A mice that had already developed anti-factor VIII. Initially, all the hemophilia A mice had been injected three times with 0.2μg factor VIII and the level of anti-factor VIII was determined by ELISA. The control mice then received three additional injections of factor VIII while the remaining mice were given mCTLA4-Ig at the same time as they received the first of three additional factor VIII injections. While many mice died of bleeding complications during this experiment because of the repeated injections and blood sample collections, the results were clearly different for the two groups.

In this Example, all mice initially received 3 intravenous injections of 0.2μg FVIII at 2 week intervals. Control mice (open circles) were then injected with factor VIII three more times and blood samples were obtained for assay (upper panel). The other mice (solid circles) were given mCTLA4-Ig (250μg, intraperitoneally) the day before and the day after the 4th factor VIII injection (as indicated by the arrow), followed by two more injections of factor VIII alone at 3 week intervals. The number of factor VIII injections prior to the blood sample tested for anti-factor VIII is indicated on the horizontal axis.

An increase in the anti-factor VIII titer was noted after the 4th injection of factor VIII for the control mice, with the mean titer going from 16 to 230μg/ml (Figure 8A). After the 5th factor VIII injection, anti-factor VIII titers were all over 350μg/ml in the four remaining control mice. In contrast, mice treated with mCTLA4-Ig at the 4th factor VIII injection had minimal or no increases in anti-factor VIII (Figure 8B and C). The administration of mCTLA4-Ig inhibited this secondary immune response to factor VIII for mice that had already developed relatively high anti-factor VIII levels, corresponding to inhibitor titers of 5-90 BU (Qian J, et al. Thromb Haemost 81 :940, 1999) (Figure 8C) as well as for mice with minimal anti-factor VIII after the three initial injections, less than 5 BU (Figure 8B).

Example 6. Determination of the role of B7-1 and B7-2 in the primary immune response to factor VIII.

The roles of B7-1 and B7-2 co-stimulatory ligands on antigen presenting cells were then evaluated, since it was their interaction with CD28 that was presumed to be prevented in the experiments using mCTLA4-Ig. To do this we crossed hemophilia A mice with B7- $1^{-/-}$ and B7-$2^{-/-}$ mice (Borriello F, et al. B Immunity 6:303, 1997; Freeman GJ et al, Science 262:907, 1993) and mice deficient in both factor VIII and either B7-1 or B7-2 were selected by genotype analysis. The hemophilia A/B7-$1^{-/-}$ and hemophilia A/B7-2 $^{-/-}$ mice were then injected intravenously with 0.2μg human factor VIII at two week intervals. Serum samples for anti-factor VIII assay were obtained 12 days after the 2^{nd} and 6^{th} factor VIII injections. After four injections, all nine hemophilia A/B7-1 $^{-/-}$ mice (open circles) had developed anti-factor VIII, with titers over 350μg/ml and a mean inhibitor level of 712 BU (Figure 9), values similar to those for otherwise normal hemophilia A mice injected with factor VIII (Qian J, et al. Thromb Haemost 81:940, 1999). In contrast, none of the eight hemophilia AlB7-$2^{-/-}$ mice (closed circles) had detectable anti-factor VIII. Similar results were obtained in hemophilia A mice treated with anti-B7-1 and anti-B7-2 antibodies.

To evaluate the T cell response of these B7-1 and B7-2 deficient hemophilia A mice, spleen cells were obtained three days after a fifth intravenous injection of factor VIII. Pooled spleen cells from 3 mice were used to establish the proliferation data. The open and closed squares in Figure 10 are for cells from untreated hemophilia A/B7-$1^{-/-}$, and B7-$2^{-/-}$ mice, respectively. The open circles are for cells from hemophilia A/B7-$1^{-/-}$ mice that received 5 intravenous injections of FVIII and the solid circles are for hemophilia A/B7-$2^{-/-}$ mice that received 5 intravenous injections of factor VIII. The concentration of factor VIII in the cultures is indicated on the horizontal axis. The T cell proliferative activity determined by 3H-thymidine incorporation showed a factor VIII dose dependent response for cells from the hemophilia/A B7-$l^{-/-}$ mice (Figure 10). In contrast, no T cell response was detected at any factor VIII level for spleen cells from hemophilia A/B7-$2^{-/-}$ mice. Thus, B7-2 has the major role in supporting an immune response to factor VIII injected intravenously, and anti-factor VIII formation is prevented if it is missing.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific compositions and methods described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

What is claimed is:

1. A composition comprising a first agent which promotes hemostasis and a second agent which inhibits a costimulatory signal in a T cell.

2. The composition of claim 1, further comprising a pharmaceutically acceptable carrier.

3. The composition of claim 1, wherein the first agent is factor VIII.

4. The composition of claim 1, wherein the first agent is a B-domain deleted variant of factor VIII.

5. The composition of claim 1, wherein the first agent is factor IX.

6. The composition of claim 1, wherein the first agent is Von Willebrand factor.

7. The composition of claim 1, wherein the second agent is a soluble form of a costimulatory molecule.

8. The composition of claim 7, wherein the second agent is a soluble form of CTLA4.

9. The composition of claim 7, wherein the second agent is a soluble form of B7-1, a soluble form of B7-2, or a combination of said soluble form of B7-1 and said soluble form of B7-2.

10. The composition of claim 8, wherein the second agent is CTLA4Ig.

11. The composition of claim 9, wherein the second agent is B7-1Ig or B72Ig.

12. The composition of claim 1, wherein the second agent is a soluble form of CD40 or CD40L.

13. The composition of claim 1, wherein the second agent is an antibody which binds to a costimulatory molecule.

14. The composition of claim 13, wherein the second agent is selected from the group consisting of an anti-B7-1 antibody, anti-B7-2 antibody, and a combination of an anti-B7 antibody and an anti-B7-2 antibody.

15. The composition of claim 13, wherein the antibody is a non-activating form of an anti-CD28 antibody.

16. A method of treating a hemostatic disorder in a subject comprising administering to the subject the composition of any of claims 1-15, such that a hemostatic disorder is treated.

17. The method of claim 16, wherein the subject has a preexisting immune response to the first agent.

18. The method of claim 16, wherein the subject does not have a preexisting immune response to the first agent.

19. The method of claim 16, further comprising administering a composition comprising an additional immunosuppressive agent.

20. The composition of claim 16, wherein the hemostatic disorder is selected from the group consisting of hemophilia A, hemophilia B, and von Willebrand's disease.

21. A method of treating a hemostatic disorder in a subject comprising administering to the subject a first agent which promotes hemostasis and a second agent which inhibits a costimulatory signal in a T cell, such that a hemostatic disorder is treated.

22. A method of treating a hemostatic disorder in a subject comprising administering to the subject a first agent which promotes hemostasis and a second agent which inhibits a costimulatory signal in a T cell, such that the immune response to the first agent is downmodulated to thereby treat a hemostatic disorder.

23. The method of claim 21 or 22, wherein the first agent is factor VIII.

24. The method of claim 21 or 22, wherein the first agent is a B-domain deleted variant of factor VIII.

25. The method of claim 21 or 22, wherein the first agent is factor IX.

26. The method of claim 21 or 22, wherein the first agent is Von Willebrand factor.

27. The method of claim 21 or 22, wherein the second agent is a soluble form of an agent which delivers a costimulatory signal to a T cell.

28. The method of claim 27, wherein the agent is a soluble form of CTLA4.

29. The method of claim 28, wherein the agent is CTLA4Ig.

30. The method of claim 27, wherein the agent is a soluble form of B7-1, a soluble form of B7-2, or a combination of a soluble form of B7-1 and a soluble form of B7-2.

31. The method of claim 30, wherein the agent is B7-1Ig, B7-2Ig, or a combination of both B7-1Ig and B7-2Ig.

32. The method of claim 21 or 22, wherein the second agent is an antibody which binds to a costimulatory molecule.

33. The method of claim 32, wherein the second agent is selected from the group consisting of an anti-B7-1 antibody, an anti-B7-2 antibody, and a combination of an anti-B7-1 and an anti-B7-2 antibody.

34. The method of claim 32, wherein the antibody is a non-activating form of an anti-CD28 antibody.

35. The method of claim 21 or 22, wherein the hemostatic disorder is selected from the group consisting of hemophilia A, hemophilia B, and von Willebrand's disease.

36. The method of claim 21 or 22, wherein the subject has a significant titer of antibodies which bind to the first agent.

Inhibition of Primary Immune Response to FVIII

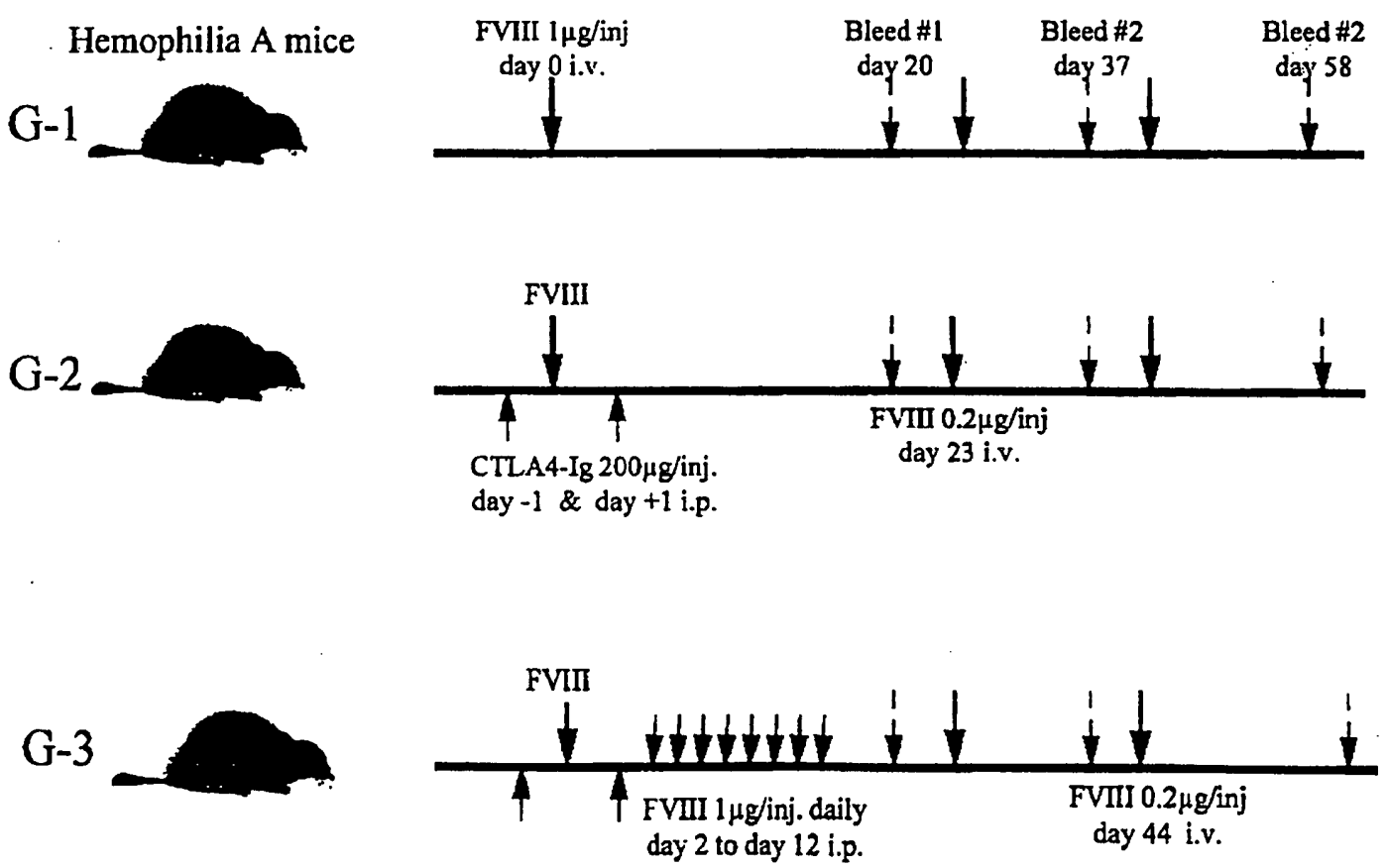


FIGURE 1

Inhibition of Immune Response to FVIII by mCTLA4-Ig in Murine Hemophilia A

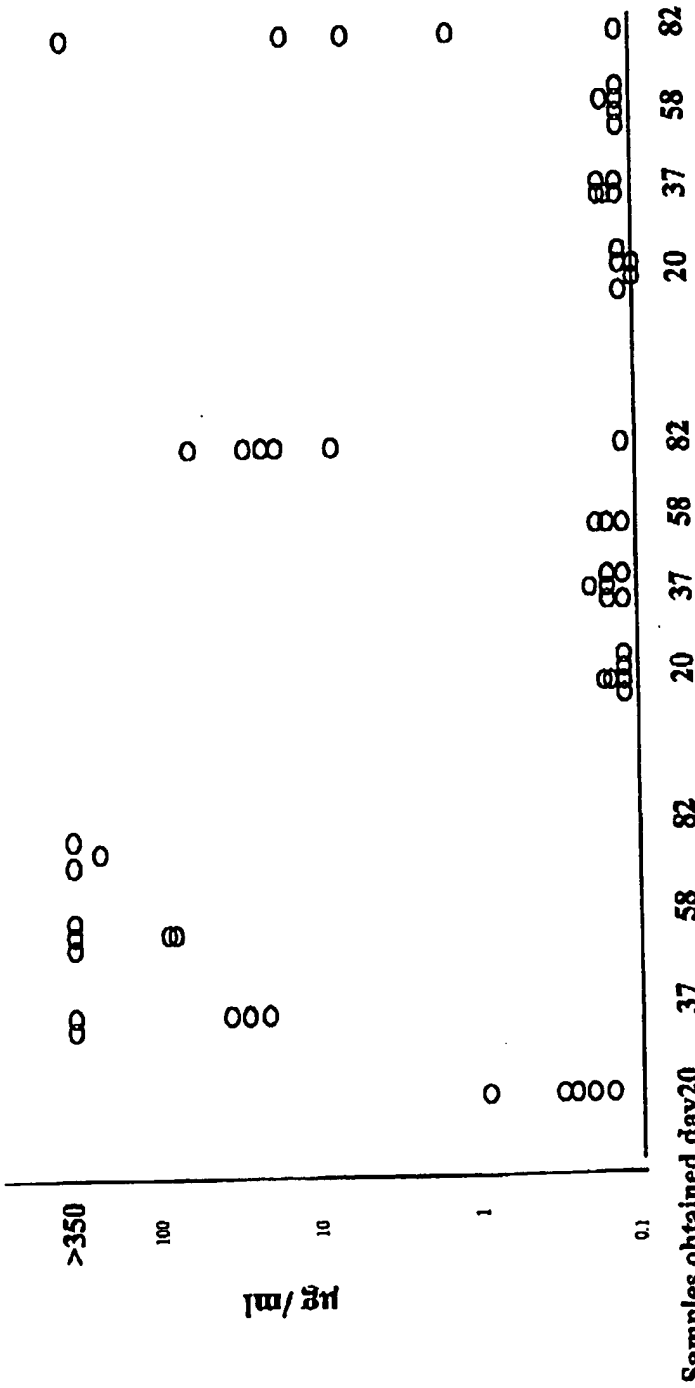
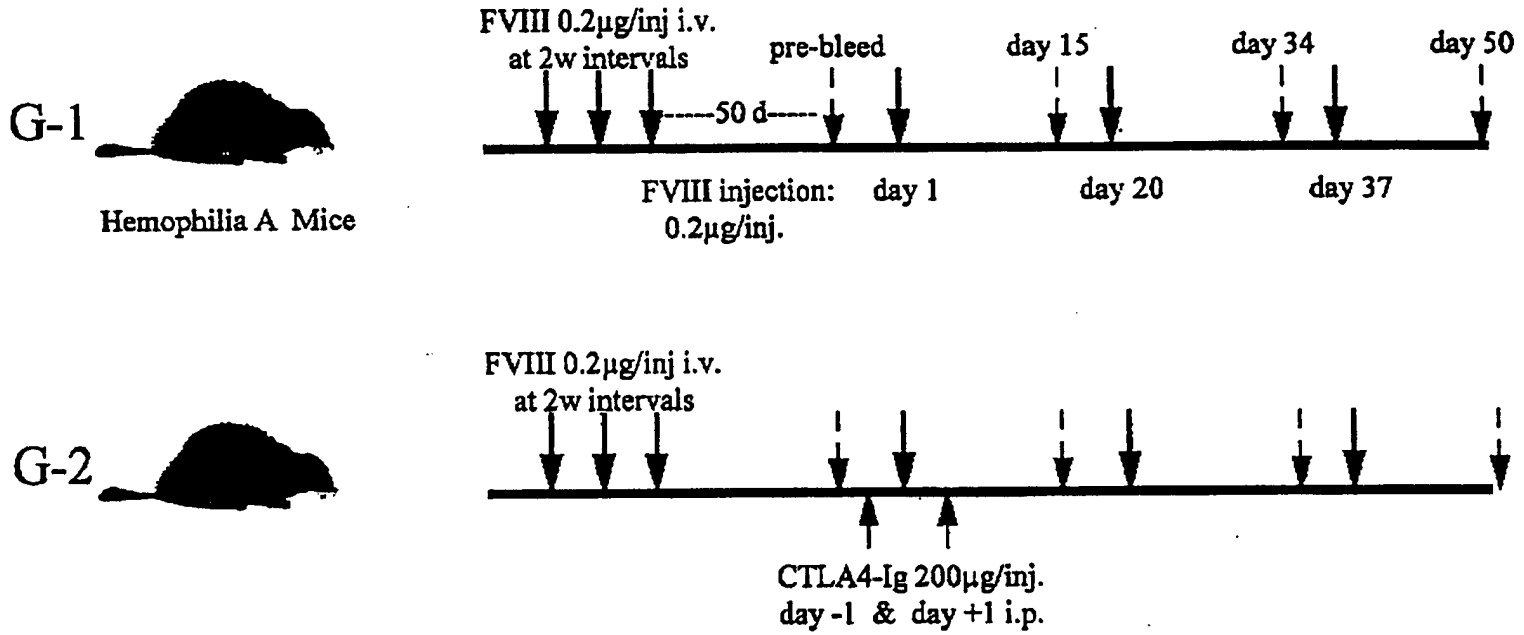


FIGURE 2

Treatment of Inhibitor Using CTLA4-Ig



secondary immune response to FVIII

FIGURE 3

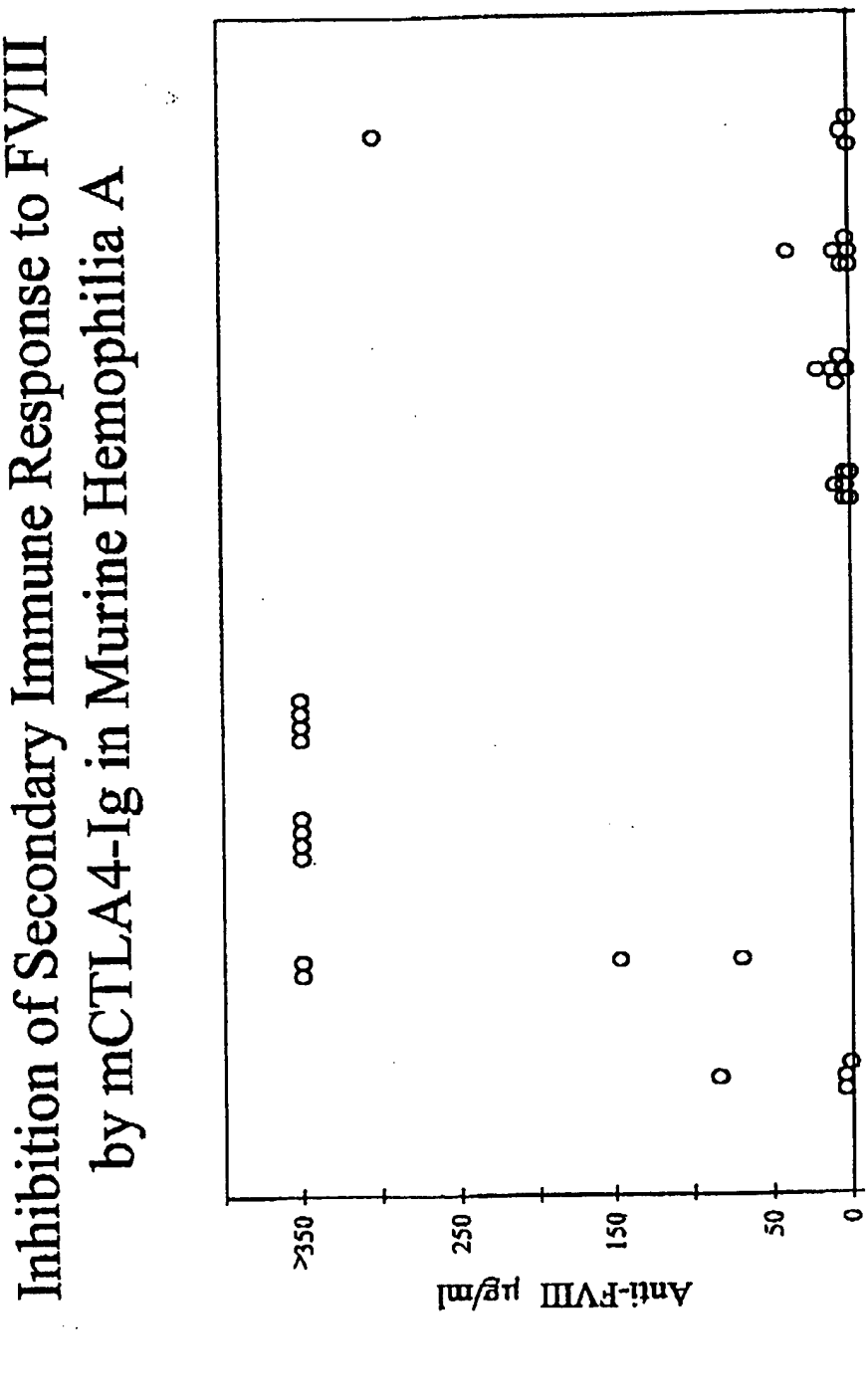

Inhibition of Secondary Immune Response to FVIII
by mCTLA4-Ig in Murine Hemophilia A
Anti-FVIII µg/ml
>350
250
150
50
0
Samples obtained day -3 15 34 50
G-1
-3 15 34 50
G-2

FIGURE 4

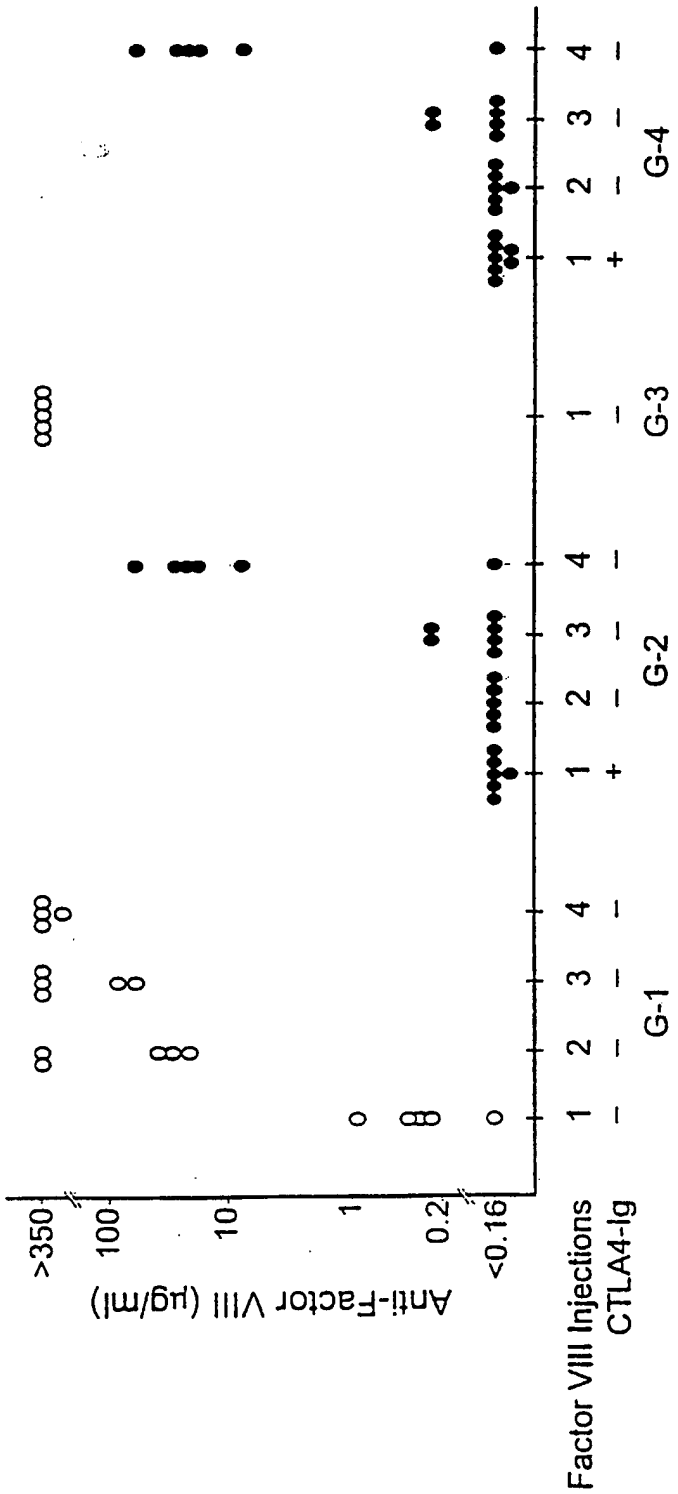

Anti-Factor VIII (µg/ml)
>350
100
10
1
0.2
<0.16
Factor VIII Injections
CTLA4-Ig
1 2 3 4 1 2 3 4 1 1 2 3 4
– – – – + – – – – – + – – –
G-1
G-2
G-3
G-4

FIGURE 5

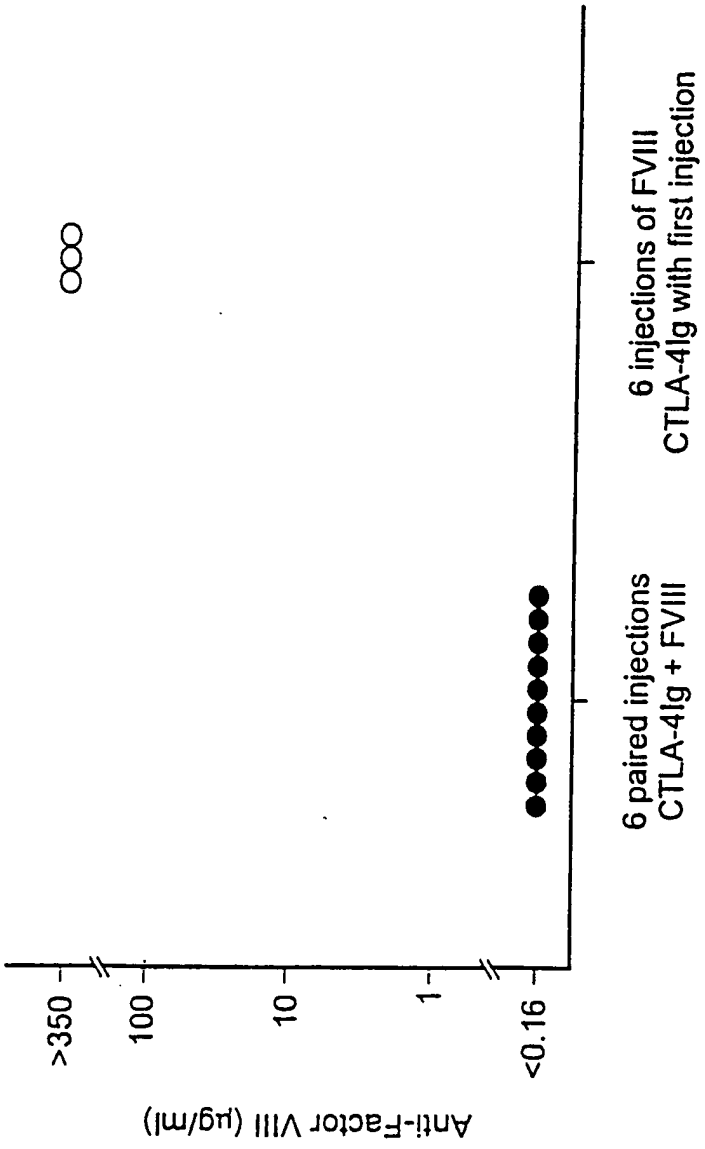

Anti-Factor VIII (µg/ml)
>350
100
10
1
<0.16
6 paired injections
CTLA-4Ig + FVIII
6 injections of FVIII
CTLA-4Ig with first injection

FIGURE 6

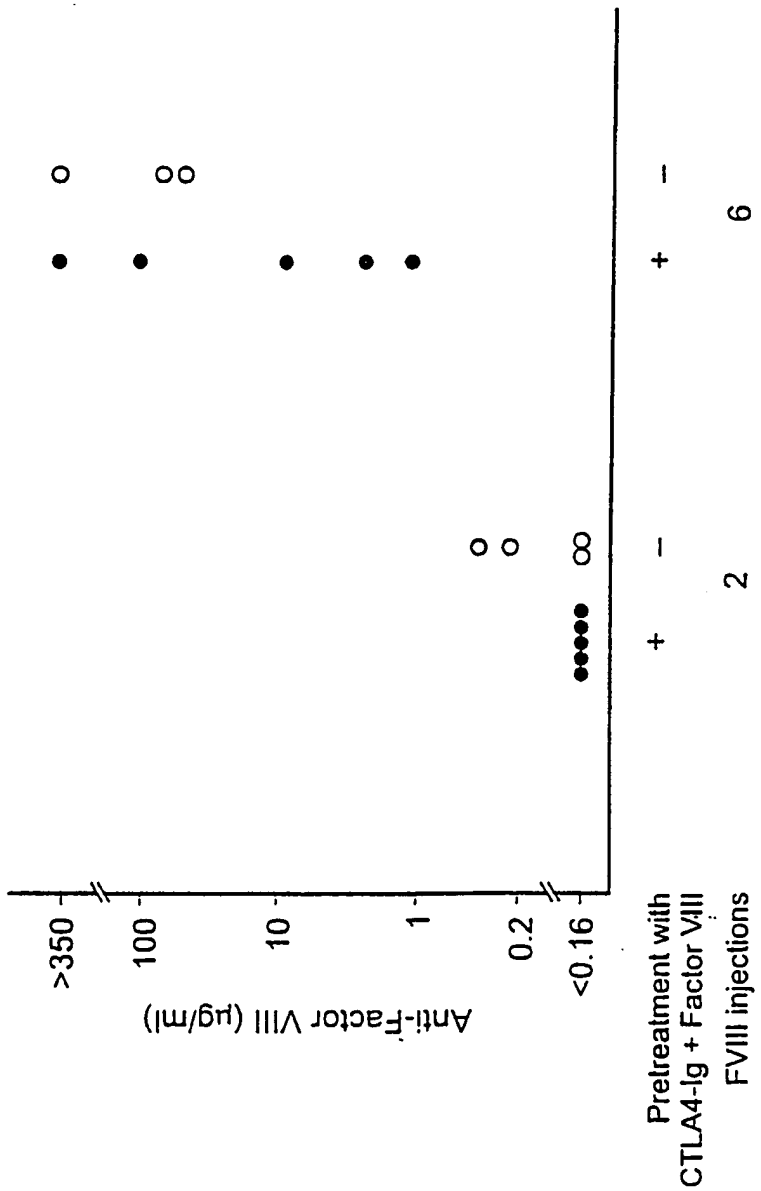

Anti-Factor VIII (µg/ml)
>350
100
10
1
0.2
<0.16
Pretreatment with CTLA4-Ig + Factor VIII
FVIII injections
+
–
+
–
2
6

FIGURE 7

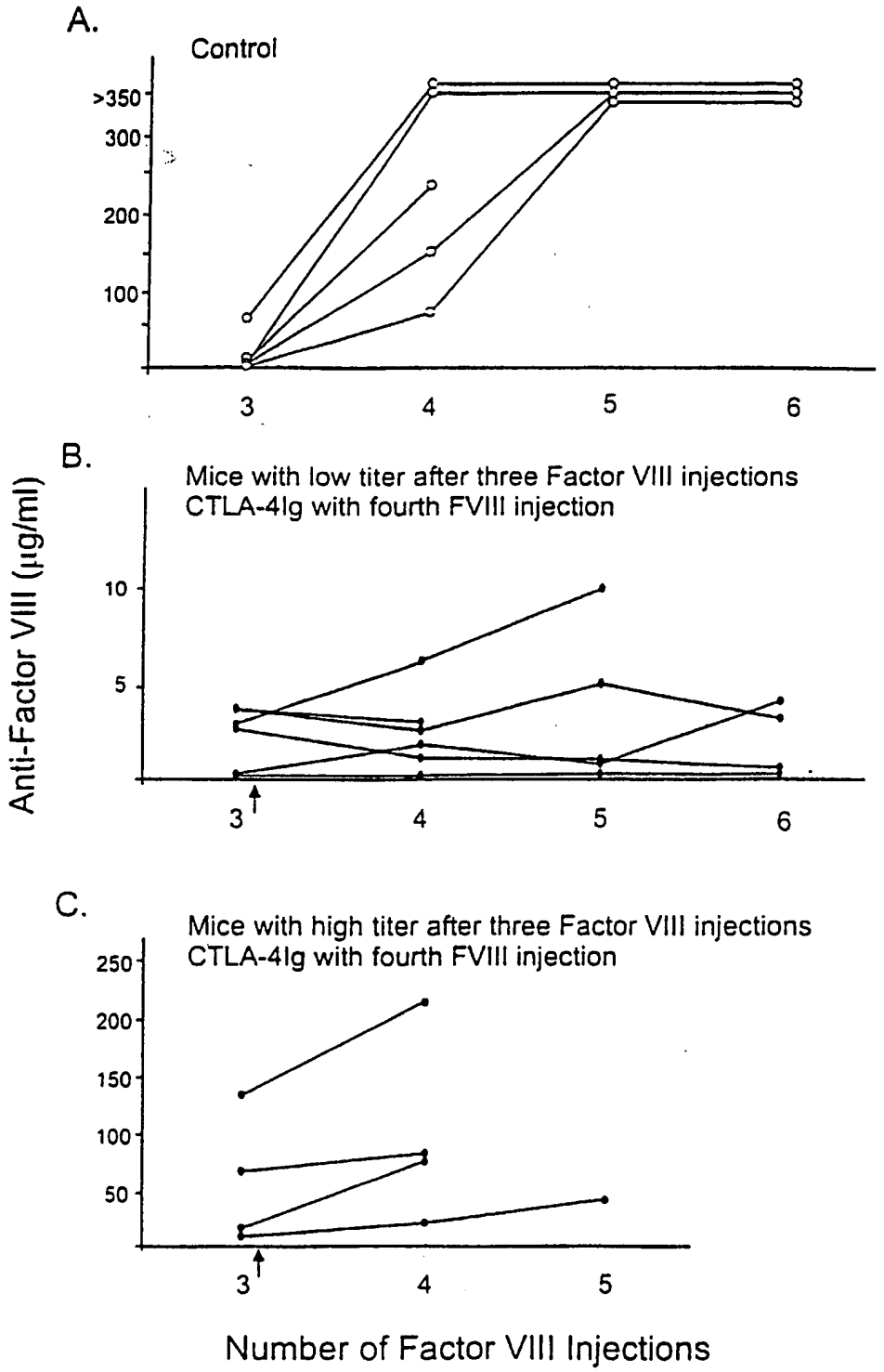

A.
Control
>350
300
200
100
3
4
5
6
B.
Mice with low titer after three Factor VIII injections
CTLA-4Ig with fourth FVIII injection
Anti-Factor VIII (µg/ml)
10
5
3
4
5
6
C.
Mice with high titer after three Factor VIII injections
CTLA-4Ig with fourth FVIII injection
250
200
150
100
50
3
4
5
Number of Factor VIII Injections

FIGURE 8

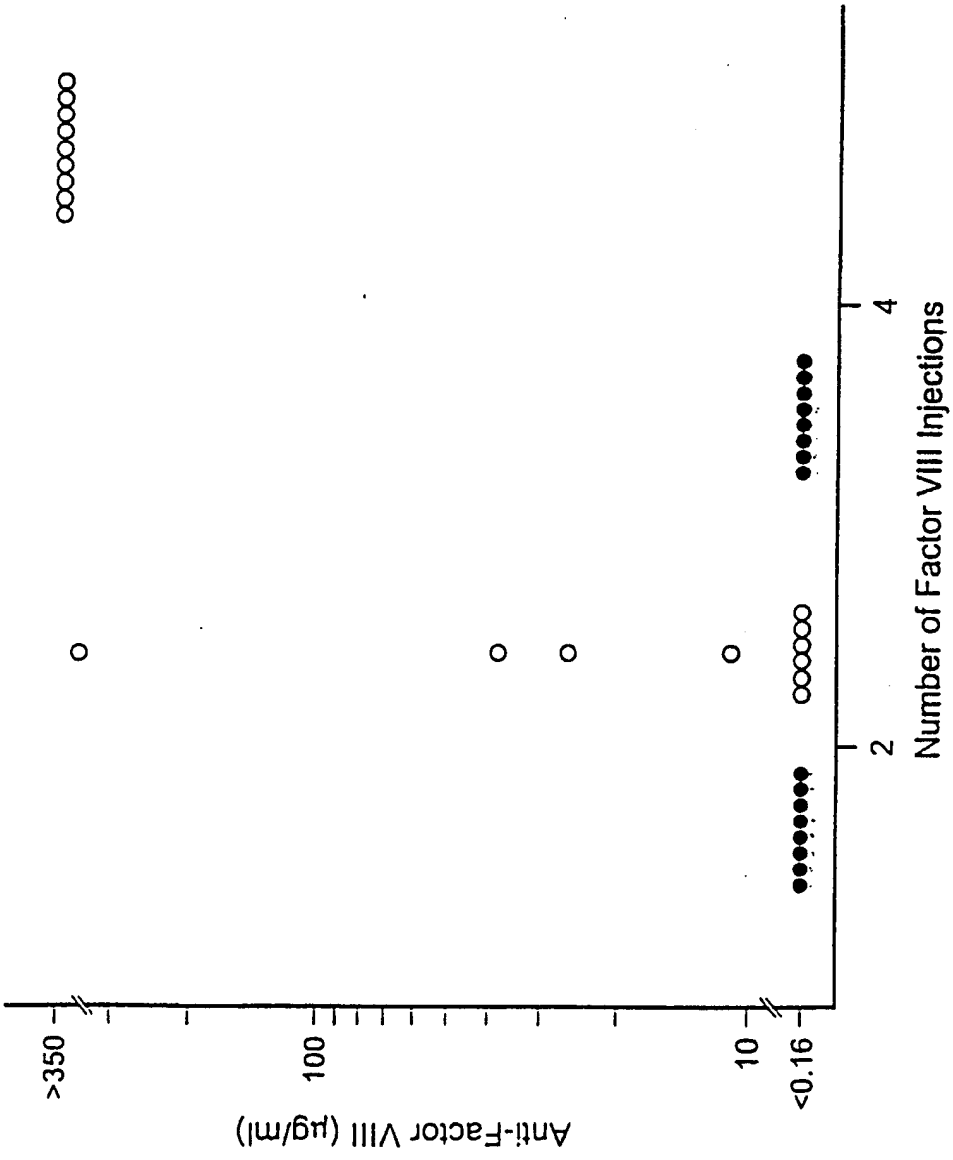

Anti-Factor VIII (µg/ml)
>350
100
10
<0.16
2
4
Number of Factor VIII Injections

FIGURE 9

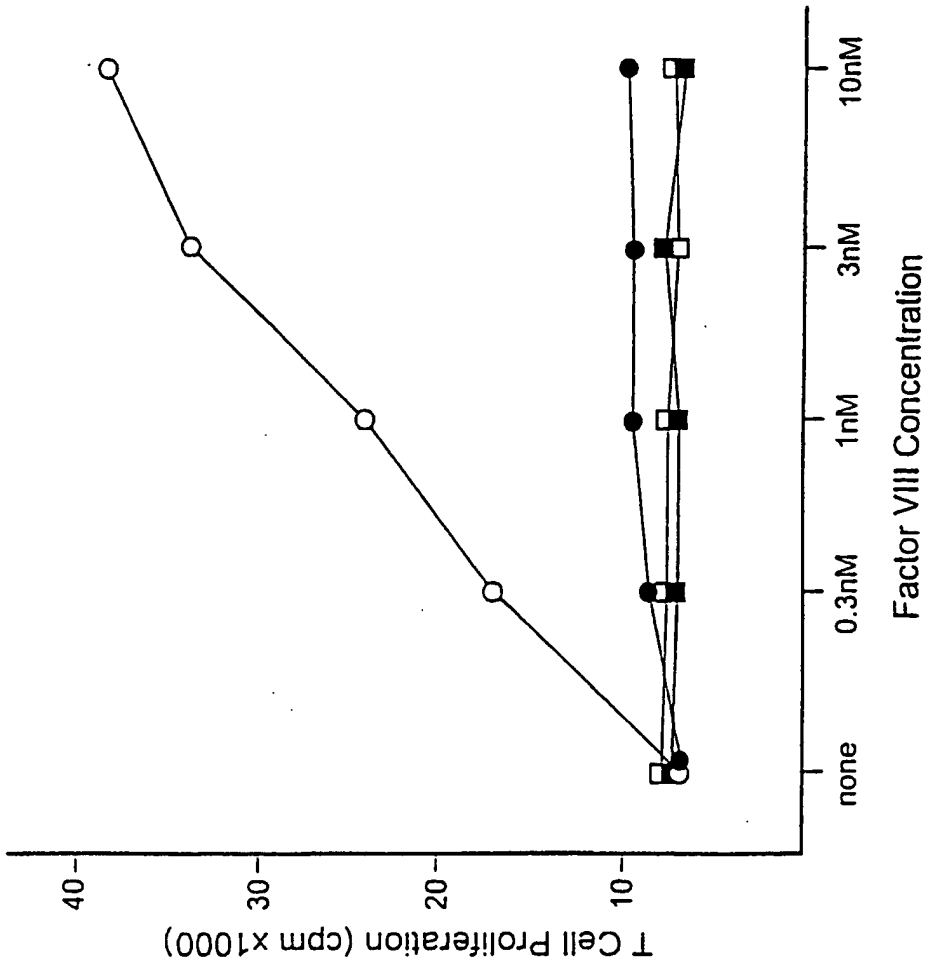

T Cell Proliferation (cpm x1000)
40
30
20
10
none
0.3nM
1nM
3nM
10nM
Factor VIII Concentration

FIGURE 10

SEQUENCE LISTING

<110> Qian, Jiahua

<120> METHODS OF DOWNMODULATING THE IMMUNE RESPONSE TO THERAPEUTIC PROTEINS

<130> GIN-013

<140> 09/158,178

<141> 1998-09-21

<160> 1

<170> PatentIn Ver. 2.0

<210> 1

<211> 6

<212> PRT

<213> Homo sapiens

<400> 1

Met Tyr Pro Pro Pro Tyr
1 5

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 99/21991

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/37 A61K38/48 A61K39/395 A61P7/04 //(A61K38/37, 38:17),(A61K39/395,38:37,38:48)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 12406 A (GENETIC THERAPY INC ;TRAPNELL BRUCE C (US); YEI SOONPIN (US); MCCL) 2 May 1996 (1996-05-02) page 11, paragraph 1 page 14, last paragraph page 16, paragraph 1 page 39 -page 45 page 47, paragraph 2 ---	1,2, 7-22, 27-36
X,P	WO 98 58672 A (ADELMAN BURT ;BIOGEN INC (US)) 30 December 1998 (1998-12-30) the whole document --- -/--	1-3,5,7, 8,10,13, 15-23, 25, 27-29, 32,34-36

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 January 2000	09/02/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Fernandez y Branas,F

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 99/21991

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category ³	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X,P	BLOOD, vol. 92, no. 10, 15 November 1998 (1998-11-15), page 709a XP000867572 abstract 2916	1-36
Y	WO 96 05860 A (NOVONORDISK AS) 29 February 1996 (1996-02-29) page 2, line 1 -page 3, line 7	1-7,9, 11-27, 30-36
Y	POTTER M.A. ET AL: "Suppression of immunological response against a transgene product delivered from micrencapsilated cells" HUMAN GENE THERAPY, vol. 9, 10 June 1998 (1998-06-10), pages 1275-1282, XP000867574 abstract page 1275, column 2, paragraph 1	1-7,9, 11-27, 30-36
A	WO 98 30241 A (BIOGEN INC ;KALLED SUSAN L (US); THOMAS DAVID W (US)) 16 July 1998 (1998-07-16) claim 24	1-36
A	WO 97 34633 A (SQUIBB BRISTOL MYERS CO) 25 September 1997 (1997-09-25) the whole document	1-36

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/21991

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 16-36 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte onal Application No
PCT/US 99/21991

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9612406 A	02-05-1996	CA 2200869 A	02-05-1996
		EP 0804076 A	05-11-1997
		JP 10507758 T	28-07-1998
WO 9858672 A	30-12-1998	AU 8153698 A	04-01-1999
WO 9605860 A	29-02-1996	AU 3161695 A	14-03-1996
		EP 0776217 A	04-06-1997
		JP 10504310 T	28-04-1998
WO 9830241 A	16-07-1998	AU 5735398 A	03-08-1998
		CZ 9902443 A	13-10-1999
		EP 0966302 A	29-12-1999
		NO 993275 A	09-09-1999
		AU 5623398 A	22-09-1998
		WO 9839026 A	11-09-1998
WO 9734633 A	25-09-1997	AU 710998 B	07-10-1999
		AU 2331097 A	10-10-1997
		CA 2246352 A	25-09-1997
		EP 0892643 A	27-01-1999
		NO 984369 A	18-11-1998
		US 5916560 A	29-06-1999